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DEPARTMENT OF BIOLOGICAL AND MEDICAL SCIENCES

RIGOROSIS THESIS

Effects of exotic plant extracts on proliferation and migration of normal human dermal fibroblasts

Účinky extraktů exotických rostlin na proliferaci a migraci primárních dermálních fibroblastů

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„Prohlašuji, že tato práce je mým původním autorských dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla použita k získání jiného nebo stejného titulu.“

V Plzni ……..…… 2018 Mgr. Zdeňka Lehečková

Contents

1 Abstract

Background: Wound healing is a physiological and highly organized complex process leading to tissue repair after an injury. A dynamic interplay between cellular and extracellular components involved in the repair process is essential for regular wound healing, results in a restoration of tissue integrity. Samoa Islands in the South Pacific are considered one of the most preserved places in the world. Local exotic plants are widely used by indigenous people to treat various skin injuries. However, the healing skills of these traditionally used plant species have been poorly studied from a scientific point of view. ***Methods:*** We analysed the effects of 16 Samoan plant extracts for their potential wound healing properties, by assessing dermal fibroblast proliferation and migration. For the evaluation of these cellular events *in vitro* DNA quantification and scratch wound assay were employed. ***Results:*** Screening of all extracts showed various effect on cell proliferation and migration with a concentration dependence. Particularly, at the highest concentration 512 μg/ml were cytotoxic 8 extracts, while at the concetration 32 μg/ml expressively reduced fibroblast proliferation 3 extracts. The effects on cell migration correlated with the proliferation assay results. Based on the screening data, 3 extracts derived from plant species *Phymatosorus scolopendria, Kleinhovia hospita* and *Premna serratifolia* have been chosen for further examination at lower concentrations 1 – 16 μg/ml, and statistically analysed. Significant stimulation of *in vitro* cell proliferation and migration by the selected extracts in majority of cases was observed, whereas the most significant outcomes provided particularly *Kleinhovia hospita* extract. ***Conclusions:*** The results suggested, that selected extracts of *Phymatosorus scolopendria, Kleinhovia hospita* and *Premna serratifolia* significantly induce wound healing properties, represented by dermal fibroblast proliferation and migration, and might be used as new therapeutical agents in a potential drug development for treatment of wounds.

Keywords: wound healing, cell proliferation, cell migration, plant extracts, traditional medicine, Samoa Islands, dermal fibroblasts

Abstrakt

Cíl práce*:** Hojení ran je fyziologický, vysoce organizovaný složitý proces vedoucí k obnovení tkáně po zranění. Dynamická souhra mezi buněčnými a extracelulárními komponentami zapojenými do tohoto procesu je zásadní pro správné hojení ran, které má za následek obnovení integrity tkáně. Samojské ostrovy v Jižním Pacifiku jsou považovány za jedno z nejzachovalejších míst planety. Místní exotické rostliny jsou široce používány původními obyvateli k léčbě různých kožních poranění. Léčivé účinky těchto tradičně používaných druhů rostlin jsou nicméně z vědeckého hlediska málo prostudovány. ***Metody: Analyzovali jsme účinky 16-ti samojských rostlinných extraktů pro jejich potenciální hojivé vlastnosti pomocí hodnocení proliferace a migrace dermálních fibroblastů. Pro vyhodnocení těchto buněčných dějů byly použity *in vitro* kvantifikace DNA a „scratch wound" test. ***Výsledky:*** Screening všech extraktů ukázal různé účinky na proliferaci a migraci buněk s patrnou koncentrační závislostí. Konkrétně bylo při nejvyšší koncentraci 512 μg/ml cytotoxických 8 extraktů, zatímco při koncentraci 32 μg/ml výrazně redukovaly proliferaci fibroblastů 3 extrakty. Účinky na migraci buněk korelovaly s výsledky zkoušek proliferace. Na základě screeningových dat byly pro další hodnocení vybrány 3 extrakty z rostlinných druhů *Phymatosorus scolopendria, Kleinhovia hospita* a *Premna serratifolia,* které byly následně testovány při nižších koncentracích 1 - 16 μg/ml a statisticky analyzovány. Po ovlivnění vybranými extrakty byla ve většině případů pozorována stimulace *in vitro* buněčné proliferace a migrace, statisticky významné výsledky poskytl zejména extrakt *Kleinhovia hospita*. ***Závěry:*** Výsledky naznačily, že vybrané výtažky z *Phymatosorus scolopendria, Kleinhovia hospita* a *Premna serratifolia* významně podporují hojivé vlastnosti, reprezentované proliferací a migrací dermálních fibroblastů, a mohly by být použity jako potenciální terapeutické látky ve vývoji nových přípravků pro léčbu ran.

Klíčová slova: hojení ran, buněčná proliferace, buněčná migrace, rostlinné extrakty, tradiční medicína, Samojské ostrovy, dermální fibroblasty

2 Introduction

Skin plays a crucial role in the human body, acting as a barrier to external noxae and pathogens. Once this barrier is disrupted, skin is not able to adequately perform the function of protection, therefore it is essential to restore promptly tissue integrity. A normal wound healing involves a series of dynamic and overlapping processes. Generally wound healing is divided into four phases known as haemostasis, inflammation, cell proliferation and tissue remodeling (Shaw and Martin 2009; Delavary, van der Veer et al. 2011).

In order to re-establish homeostatic mechanisms and minimise fluid loss, multiple parallel and mutually related pathways are activated to induce tissue restoration (Greaves, Ashcroft et al. 2013). An injured skin exposes underlying tissue to outside environment, providing an open access to infection, that often results in alterations of wound repair process, leading to delayed wound healing, chronic wounds, or excessive scarring (Shaw and Martin 2009; Singh, Young et al. 2017). The coordinated interplay between cellular and extracellular components of intricate signaling networks is necessary for proper wound healing. Modulation of diverse growth factors, chemokines and cytokines influences cell proliferation, migration, adhesion, extracellular matrix (ECM) production and other metabolic activities involved in tissue repair. The culmination of these complex biological courses results in the replacement of former skin structures, leading to a scar formation (Rodrigues and Longaker 2000; Greaves, Ashcroft et al. 2013). The main cellular actors in these restoring processes are dermal fibroblasts, performing migration into wound bed followed by their proliferation. This thesis is focused on the investigation of these fibroblast events as two pivotal aspects in wound healing process.

3 Aims

The aim of the thesis is *in vitro* characterization of the wound healing properties of tested plant extracts, assessing the effects on proliferation and migration of human dermal fibroblasts, which actively participate in wound healing process. The analysis was divided into two steps.

1) Screening of 16 extracts was performed by means of fibroblast proliferation and migration. Proliferation was measured by DNA quantification and migration was assesed by scratch wound assay.
2) 3 extracts with the best promising effects on fibroblast proliferation and migration were selected and analysed at lower concentrations suitable for therapeutical application.

4 Review of literature

4.1 Wound healing

Wound healing is an essential physiological process that involves an organization of various cells, chemical signs and other components to repair a tissue. The aim of the process is to obtain functional and esthetically satisfactory substitution of tissue, a scar (Mendonça and Coutinho-Netto 2009).

Skin is a complex tissue, and particularly full thickness wounds can cause damage to many structures of the skin (Shaw and Martin 2009). There are three main skin layers, that includes a thin outer barrier, the epidermis, a thicker connective tissue, the dermis, and subcutaneous layer underneath the dermis, the hypodermis (Fig. 1). While the epidermis has mainly protective function of skin, the dermis is a complicated structure consisting of fibroblasts, ECM, nerves, blood and lymphatic vessels, and associated epidermal appendages such as hair follicles, sweat and sebaceous glands. The dermis layer is besides other important for sensation, protection and thermoregulation. The adipose tissue of hypodermis has in particular metabolic function. A wound can also cause damage at the level of individual cells or specific organelles (Shaw and Martin 2009; Monfort, Soriano-Navarro et al. 2013).

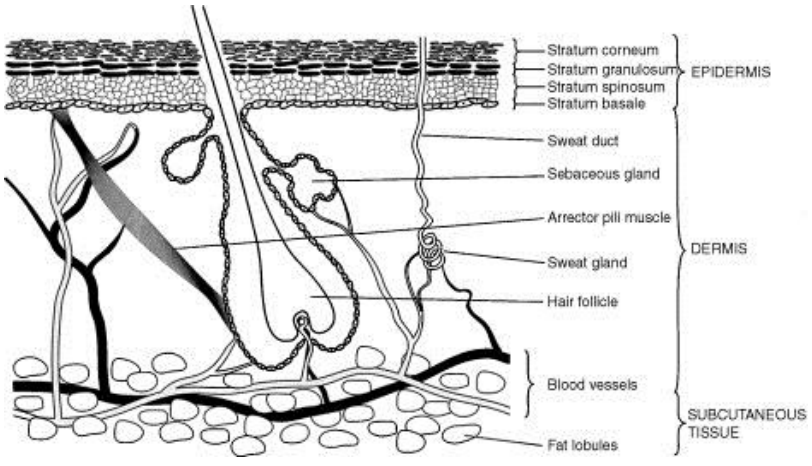


Fig. 1. ***Diagram of skin layers.*** Adapted from (El Maghraby, Barry et al. 2008).

Tissue regeneration and repair processes occur after the appearance of the lesion, according to a specific pathological condition. The stimuli causing lesions, can be external or internal

(Gonzalez, Costa et al. 2016). Disruptions can be caused inter alia due to a physical, chemical, electric, thermal, infectious or immunological agents (Shah and Amini-Nik 2017). Regardless created by any stimuli, the lesion breaks physical continuity of functional tissue (Gonzalez, Costa et al. 2016).

Normal wound healing processes follow specific time sequences and can be generally categorized into four phases, that is haemostasis, inflammation, proliferation and tissue remodeling (Fig. 2). Many factors can interfere with this process, resulting in delayed wound healing, chronic wounds and poor cosmetic outcome (Singh, Young et al. 2017). For each phase are predominant different cell types, cytokines and chemokines. The particular phases of wound healing, however, are not completely seperated but mutually overlapping in time (Portou, Baker et al. 2015). Immediately after skin injury, a temporary repair is achieved in the form of a clot. The clot plugs a defect, and subsequent steps to regenerate the missing parts are initiated. Inflammatory cells, fibroblasts and new capillaries overrun the clot and form a contractile granulation tissue. At the end of wound healing, during the maturation phase, collagen becomes more organised, increasingly cross-linked strengthened, and ultimately forms the mass of a mature scar (Rodrigues and Longaker 2000).

However, there are many breakpoints in the healing process, which can lead to unsatisfactory result. That is why, it is neccesary to have greater understanding of the biochemical mechanisms involved in the healing of wounds and tissue regeneration (Mendonça and Coutinho-Netto 2009).

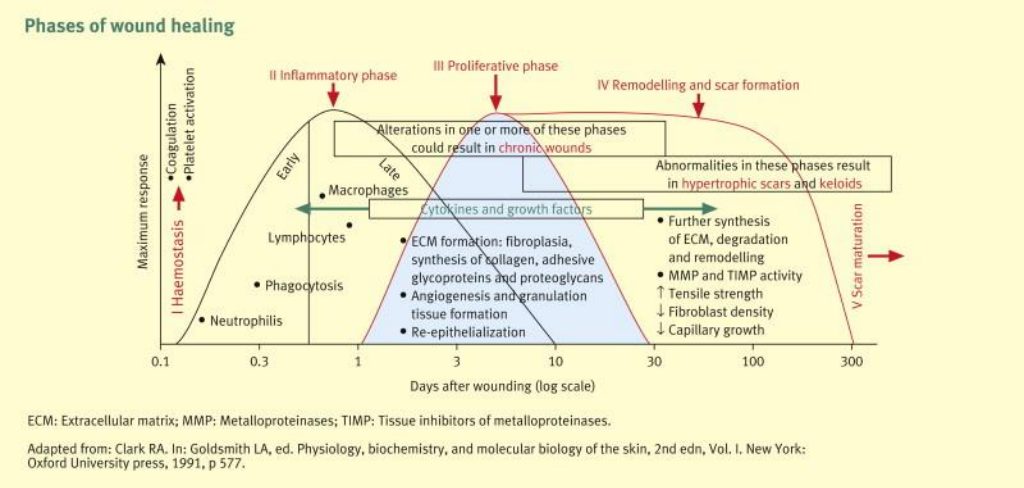


Fig. 2. ***Phases of wound healing.*** Adapted from (Enoch and Leaper 2008).

4.1.1 Haemostasis

First of all, immediately after the injury, the response of body is to prevent exsanguination and promote haemostasis (Harper, Young et al. 2014). Process of haemostasis is composed of three major steps, vasoconstriction, platelet plug formation and blood coagulation. Platelets aggregate at the site of injury, while haemostasis is achieved with ongoing local vasoconstriction and activation of clotting cascade, that results in fibrin clot formation (Portou, Baker et al. 2015).

Under physiological conditions, platelets circulate in vessels, and healthy vascular wall provides a natural barrier to unintentional activation of platelets, leading to formation of platelet plug. An important role play inhibitory mediators such as nitric oxide and prostacyclin, releasing from the intact endothelium. Platelets become activated when the continuity of the endothelial layer is disrupted, and the underlying subendothelial tissue is exposed (Golebiewska and Poole 2015).

4.1.1.1 Vasoconstriction

Damaged arterial vessels rapidly constrict through the contraction of smooth muscle in the vessel wall, mediated among others by increasing cytoplasmic calcium levels (Harper, Young et al. 2014). Reflex vasoconstriction is responsible for an initial slowing down of blood flow to the injured area. The reduced blood flow enables contact activation of platelets and coagulation factors. The vasoactive amines such as serotonin and adrenalin, thromboxane A2 produced by platelets, and fibrinopeptides cleaved during fibrin formation also influence vasoconstrictive activity (Hakim and Canelo 2007).

4.1.1.2 Platelet plug formation

Platelets adhere to damaged endothelium to form a platelet plug, so called *primary haemostasis*. Adhesion is mediated chiefly by Von Willebrand factor (vWF), interacting with collagen and platelet glycoprotein receptors. When platelets come across the injured endothelium cells, they change its shape, and a series of biochemical steps leads to the platelet activation. Activated platelets release cytoplasmic granules such as adenosine diphosphate (ADP), thromboxane A2, fibrinogen, and other activating factors e.g. collagen and thrombin, stimulate platelet aggregation and activation of other platelets (Fig. 3). All of these mechamisms lead to the formation of a platelet plug. The process is reversible, reflecting a positive feedback loop. Platelets play a major role in the entire haemostatic process, particularly in *primary haemostasis* (Clemetson 1999; de Queiroz, de Sousa et al. 2017).

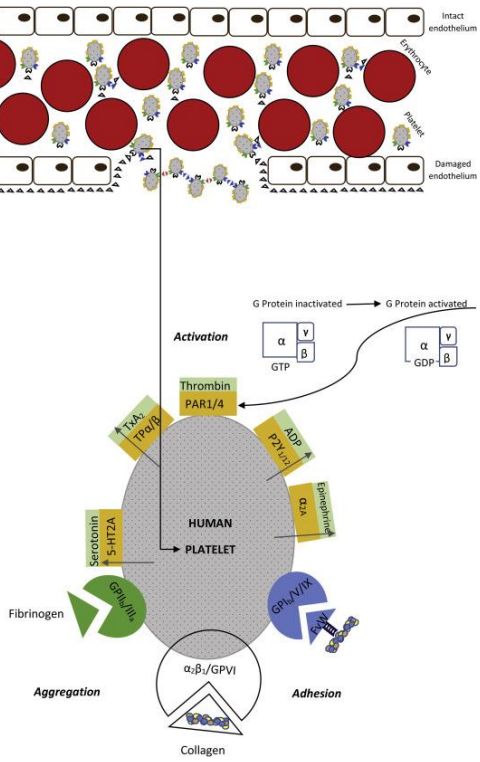


Fig. 3. ***Schematic diagram of adhesion, activation and platelet aggregation after vascular injury.*** Adapted from (de Queiroz, de Sousa et al. 2017). ADP, Adenosine diphosphate; FvW, Von Willebrand factor; GDP, Guanosine diphosphate; GTP, Guanosine triphosphate; TxA_2, Thromboxane A2

4.1.1.3 Blood coagulation

Once the platelet plug has been formed, the clotting factors are activated in a sequence of events known as *coagulation cascade* (Fig. 4). That leads to the formation of fibrin from inactive fibrinogen plasma protein, catalysing by serine protease thrombin. The fibrin meshwork strengthens the platelet plug and makes it insoluble. This process is referred as *secondary haemostasis* (Sepúlveda, Palomo et al. 2015).

Coagulation, leading to the formation of a clot, is achieved through three key mechanisms:

- Extrinsic pathway of the clotting cascade (tissue factor pathway) – tissue damage results in exposure of tissue factor, thromboplastin, to circulating blood. This is responsible for activation of factor VII, which in turn, activates factor X. The extrinsic pathway is important especially for the initiation of blood coagulation.

- Intrinsic pathway of the clotting cascade (contact activation pathway) – endothelial damage exposes the subendothelial tissues to blood, which results in the activation of factor XII (Hageman factor). This initiates the proteolytic cleavage cascade which results in the activation of factor X.
  Reaction of factor XII with prekallikrein and high molecular weight kininogen leads to activation of factor XI, that activates factor IX. Activated factor IX, in association with Ca^{2+}, factor VIIIa and platelet phospholipids, activates factor X. The main role of intrinsic pathway is in amplification of coagulation.

- Final common pathway – both extrinsic and intrinsic pathways result in activated factor X. In association with factor V as a cofactor, phospholipids and Ca^{2+}, activated factor X converts inactive plasmatic protein prothrombin to thrombin. Thrombin, as a catalysing enzyme, cleaves soluble fibrinogen to fibrin, polymerasing into fibrin meshwork. In the end the fibrin clot is stabilized via activated factor XIII, which forms covalent bonds, that crosslink the fibrin polymers (Hakim and Canelo 2007; Singh, Young et al. 2017)

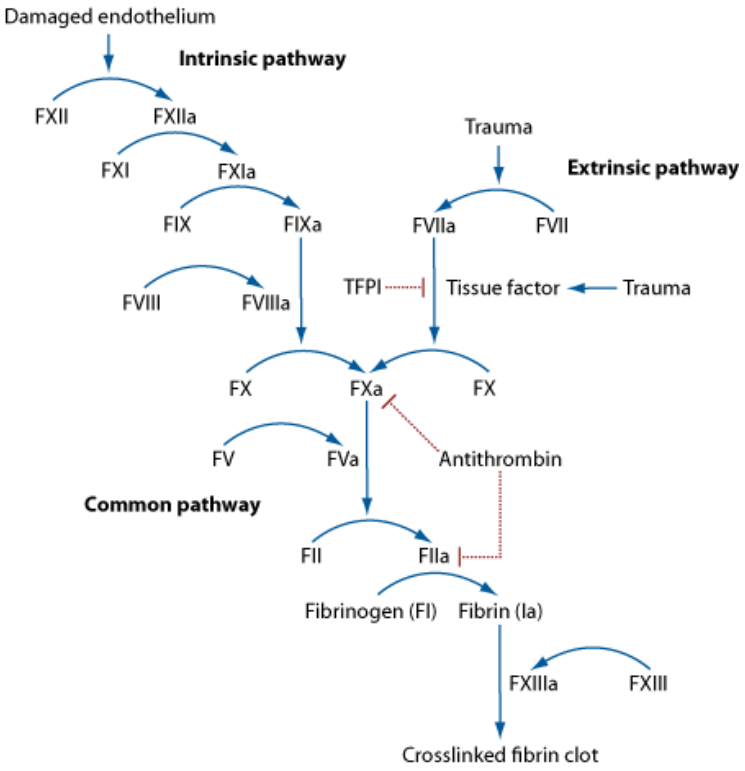


Fig. 4. ***Coagulation cascade.*** Adapted from (http://www.hemophiliareport.com/sung.php, 10th February 2018).

4.1.1.4 Platelets and wound healing

Platelets have a crucial role in wound healing process. They are not only essential for the clot formation, they also produce multiple growth factors and cytokines, which are important in regulation of the healing cascade. Over 300 signaling molecules have been isolated from activated platelets. The main platelet derived molecules are referred in Table 1 (Harper, Young et al. 2014). Secreted growth factors and cytokines diffuse into the surroundings to recruit neutrophils and macrophages, and to stimulate resident stem cells, endothelial cells, osteoblasts, fibroblasts and epidermal cells. Bound to the cell surface receptors, they result in the activation of intracellular signaling cascades, leading to migration, proliferation and differentiation of cells (Fernandez-Moure, Van Eps et al. 2017). Besides that, from the platelet cell membrane, is released arachidonic acid, which breaks down into a number of potent molecules such as prostaglandins, leukotrienes and thromboxanes, having an important role in stimulating the inflammatory response (Harper, Young et al. 2014).

Table 1. ***Platelet growth factors involved in wound healing.*** Adapted from (Harper, Young et al. 2014).

Growth factor	Action
TGF-α	Formation of granulation tissue Stimulates proliferation of epithelial cell and fibroblasts
TGF-β	Chemotaxis Transdifferentiation of fibroblasts to myofibroblasts Collagen matrix construction Stimulation of angiogenesis Wound contraction Release of other growth factors Metalloproteinases stimulation
PDGF	Chemotaxis Fibroblast proliferation Collagen deposition
VEGF	Stimulate angiogenesis Neovascularization
Serotonin	Vasoconstriction Platelet Aggregation Chemotaxis Increase vascular permeability
TNF-α	Chemotaxis Nitric oxide release Activation of other growth factors
Thromboxane A_2	Platelet aggregation Vasoconstriction
Leukotrienes	Increased vascular permeability Chemotaxis Leukocyte adhesion
Interleukin-1	Chemotaxis
Lipoxins	Decrease inflammatory response Inhibit chemotaxis (neutrophils)

PDGF, platelet derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

4.1.1.5 Fibrinolysis

Eventually, blood clots are reorganised and resorbed by a process known *fibrinolysis* (Fig. 5). Fibrinolytic system regulates the breakdown of blood clots to restore a vascular integrity (Monagle and Massicotte 2011). The maintenance of an equilibrium between coagulation and

fibrinolysis is essential, as imbalance leads to abnormal bleeding or increased risk of thrombosis.

Under physiological conditions, fibrinolytic process is achieved through two key mechanisms, activation of plasminogen to plasmin, and fibrin breakdown by plasmin into fibrin degradation products. These steps lead to dissolving of the thrombus (Gue and Gorog 2017). The process is parallel to the time frame of clot fomation (Hakim and Canelo 2007).

Plasminogen converts to plasmin by tissue plasminogen activator (t-PA), creating a plasminogen t-PA complex. Formation of this complex is dependent on lysine-binding sites on plasminogen, and t-PA and carboxyl-terminal lysines on fibrin. Urokinase plasminogen activator (u-PA) can also form a complex with plasminogen, however, it has a lower affinity than t-PA (Ilich, Bokarev et al. 2017).

The fibrinolytic system is regulated by several inhibitors as well, ensuring a haemostasis balance. Plasminogen activator inhibitor-1 (PAI-1) binds t-PA, resulting in an inactive complex. Under normal circumstances, the concentration of PAI-1 in plasma exceeds plasminogen activators, preventing unintentional bleeding. Alpha$_2$-antiplasmin and alpha$_2$-macroglobulin are other ways of fibrinolysis regulation, also forming inactive complexes with plasmin (Ilich, Bokarev et al. 2017).

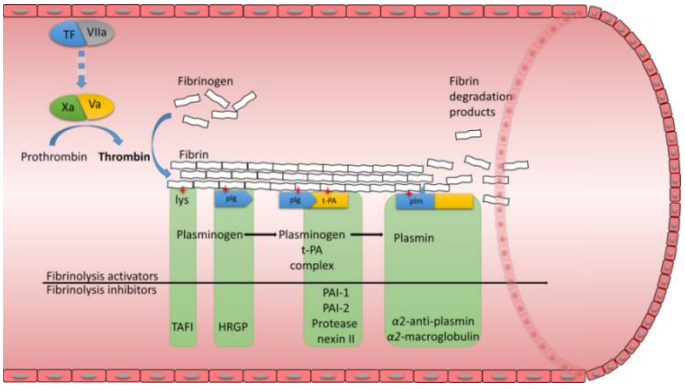


Fig. 5. ***Simplified fibrinolysis scheme.*** Adapted from (Ilich, Bokarev et al. 2017). HRGP, Hydroxyproline-rich glycoprotein; PAI-1,2, Plasminogen activator inhibitor-1,2; TAF1, TATA-box binding protein associated factor 1; TF, Tissue factor; t-PA, tissue plasminogen activator

4.1.2 Inflammatory phase

The key aim of an inflammatory phase is to prevent infection. Regardless to the etiology of the wound, the mechanical barrier is no longer intact, inclinable to invading of microorganisms (Singh, Young et al. 2017). Skin cells are exposed to acute phase signals such as damage-associated or pathogen-specific molecular patterns, causing an initiating and persisting inflammation (Sorg, Tilkorn et al. 2017). Proper wound healing is achieved by adequate activation of inflammatory cells, neutrophils and macrophages, which release pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), platelet derived growth factor (PDGF), transforming growth factor β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF) and interleukins 1, 6, 8 (IL-1,6,8) from the newly formed clot and directly from the damaged tissues. That results in the proliferation and infiltration of activated fibroblasts to the wound site. While the appropriate level of each cytokine is essential for healing, inordinate levels of inflammatory cytokines result in excessive fibroblast proliferation, leading to hypertrophic scarring (Shah and Amini-Nik 2017).

4.1.2.1 Vasodilatation

Reduced blood flow mediated by arteriole constriction leads to tissue hypoxia and acidosis. This promotes the production of nitric oxide, adenosine and other vasoactive metabolites to cause a reflex vasodilatation and relaxation of the arterial vessels. Simultaneously, histamine releases from mast cells and also contributes to increase vasodilatation. A period of vasodilation is an important mechanism by which the wound can be exposed to increased blood flow and vascular permeability, facilitating easier entry of inflammatory cells into the extracellular space around the wound. That explains the characteristic warm, red and swollen appearance of early wounds (Harper, Young et al. 2014).

4.1.2.2 Early and late cellular aspects of inflammation

Circulating neutrophils also known as polymorphonuclear leukocytes (PMN), are the early responders, begin migration within minutes from the blood into the immature wound, having a peak at 24 hours (Portou, Baker et al. 2015). PMN provide a crucial defence against microbial invasion. They have three main mechanisms for pathogen and tissue debris eradication. Firstly, they can directly ingest and destroy foreign particles by *phagocytosis*. Secondly, neutrophils degranulate and release a variety of toxic substances such as lactoferrin, proteases, neutrophil elastase and cathepsin, which liquidate microorganisms as well as dead tissue. Eventually, as a side product of neutrophil activity, oxygen free radicals are produced, having also bactericidal skills. Once the neutrophils have completed their task, they undergo apoptosis, phagocytosed by macrophages (Singh, Young et al. 2017).

Later, platelet degranulation process, activation of the complement system and the migration of neutrophils, result in the production of chemotactic factors, recruiting monocytes to the wound. Under the influence of local cytokines, monocytes differentiate in mature wound macrophages (Portou, Baker et al. 2015). Within 48-72 hours tissue macrophages become the predominant cell type in the wound. Macrophages are much larger phagocytic cells and they are able to survive in the more acidic wound environment, presented at this stage. Macrophages continue the process of wound bed clearance through phagocytosis of apoptotic cells including the early phase neutrophils, tissue debris and microbial organisms. In addition, macrophages release protease and metalloprotease enzymes, also helpful in the clearing of the wound. Besides the phagocytic role, the important function of wound macrophages is the release of proinflammatory cytokines such as growth factors, regulating the inflammatory response, stimulating angiogenesis and enhancing the granulation tissue formation. That is why macrophages are considered as a crutial factor for the transition to the proliferative phase of wound healing (Portou, Baker et al. 2015; Singh, Young et al. 2017). Lymphocytes appear in the wound after 72 hours and are important in regulating wound healing through the production of ECM and collagen remodeling. A summary of the cells involved in wound healing process is viewed in Table 2 (Singh, Young et al. 2017).

Table 2. ***Cells involved in wound healing.*** Adapted from (Delavary, van der Veer et al. 2011; Singh, Young et al. 2017).

Cell type	Time of action	Function
Platelets	Seconds	Thrombus formation Activation of coagulation cascade Release inflammatory mediators (PDGF, TGF-β, FGF, EGF, histamine, serotonin, bradykinin, prostaglandins, thromboxane)
Neutrophils	Peak at 24 hours	Phagocytosis of bacteria Wound debridement Release of proteolytic enzymes Generation of oxygen free radicals Increase vascular permeability
Keratinocytes	8 hours	Release of inflammatory mediators Stimulate neighbouring keratinocytes to migrate Neovascularization
Macrophages	48-72 hours	Phagocytosis of bacteria Wound debridement Rich source of inflammatory mediators including cytokines Stimulate fibroblast division Collagen synthesis Angiogenis
Lymphocytes	72–120 hours	Regulates proliferative phase of wound healing Collagen deposition
Fibroblasts	120 hours	Synthesis of granulation tissue Collagen synthesis Produce components of ECM Release of proteases Release of inflammatory mediators

The inflammatory phase of wound healing persist as long as there is a need for it, ensuring that all excessive pathogens and debris from the wound is cleared. However, protracted inflammation can lead to extensive tissue damage, delayed proliferation and results in a chronic wound formation. Multiple factors, including lipoxins and the products of arachidonic acid metabolism, have anti-inflammatory properties, which decrease the immune response and allow to turn in to the next phase of wound healing (Singh, Young et al. 2017).

4.1.3 Proliferative phase

The proliferative phase occurs about day 3 of wound healing and lasts up to 2-4 weeks after an injury, widely overlapping with the preceding inflammatory phase (Enoch and Leaper 2008). It is characterized by processes such as active fibroplasia, epidermal regeneration, angiogenesis and wound contraction (Delavary, van der Veer et al. 2011). With progression of the proliferative phase, the provisional fibrin matrix is replaced by the newly formed granulation tissue (Enoch and Leaper 2008). The proliferative phase involves numerous important cellular and molecular components, that contribute to ECM and granulation tissue formation. ECM provides support for further cellular influx, adhesion and differentiation. After its formation, ECM undergoes continuous synthesis and remodeling. Angiogenesis is essential to replace damaged capillaries and to restore the supply of oxygen, blood constituents and nutrients to wounded tissue (Greaves, Ashcroft et al. 2013). Granulation tissue includes inflammatory cells, fibroblasts and new blood vessel network in a matrix of fibronectin, collagen, glycosaminoglycans and proteoglycans, the components of a provisional ECM. Further fibroblasts interact with myofibroblasts and produce ECM mainly in the form of collagen, which eventually forms the mass of a tensile scar (Enoch and Leaper 2008; Geoffrey C, Sabine et al. 2008).

4.1.3.1 Fibroplasia

Fibroblasts play a crutial role in the formation of granulation tissue. The process involving fibroblasts and ECM, which they synthesise, is known as *fibroplasia*. That is influenced by numerous bioactive molecules presented in the wound bed during healing. Growth factors such as PDGF, FGF-2 and TGF-β, interacting with fibrinogen chains and thrombin, stimulate fibroblasts migration and proliferation (Enoch and Leaper 2008; Greaves, Ashcroft et al. 2013).

Fibroblasts appear in the wound 2–4 days after the wounding. Following injury, fibroblasts are attracted to the wound in particular by PDGF and TGF-β. Once they are in the wound, fibroblasts proliferate and produce the matrix proteins fibronectin, hyaluronan and later, collagen and proteoglycans. These components help to construct the new ECM, which

supports further ingrowth of cells and is essential for the repair process (Enoch and Leaper 2008). Fibronectin works as an anchor for fibroblasts and it migrates within the wound. One of the main fibroblast task is a production of collagen. The synthesis and deposition of collagen is a critical event in the proliferative phase and the wound healing in general (Greaves, Ashcroft et al. 2013). ECM consists of fibrous structural proteins such as collagen and in small amounts elastin, and an interstitial matrix mainly composed of proteoglycans and glycosaminoglycans (Enoch and Leaper 2008).

ECM provides a scaffold for cellular adhesion and migration during tissue restoration and ultimately create the architecture of the healed wound. The initially disorganised array of tentative matrix later becomes highly organized predominantly collagenous final structure (mostly collagen types I and III). The dense population of fibroblasts, macrophages and neovascularization, embed in a ECM is referred as a granulation tissue (Greaves, Ashcroft et al. 2013).

4.1.3.1.1 ECM definition

The ECM components can be divided into fiber-forming and nonfiber-forming molecules work as structural units, and matricellular proteins without structural function, modifying cell–matrix interactions (Fig. 6). Fiber-forming molecules provide the ECM structure by a framework of firm proteins, defining rigidity and elasticity of a tissue. The nonfiber-forming molecules, mostly proteoglycans and glycosaminoglycans, create a charged, dynamic, and osmotically active space. The most prevalent fiber-forming protein is collagen. Other fibrous proteins in the dermis include fibrin, fibronectin, vitronectin, elastin, fibrillin, and glycoproteins laminins and integrins. Nonfiber-forming proteoglycans and glycosaminoglycans fill in the majority of a tissue interstitial space. The most abundant proteoglycans in the skin are hyaluronan, decorin, versican, and dermatopontin. Among interacting matricellular proteins rank osteopontin, osteonectin (also known as SPARC), tenascin-C and fibulins. Unlike most ECM components, matricellular proteins can be absent in healthy tissue and expressed temporarily only after skin wounding. Fibroblasts produce the majority of these ECM components, while the same molecules simultaneously modify the fibroblast function. In this sense it is a form of autocrine regulation that is crucial in the wound healing process (Tracy, Minasian et al. 2016).

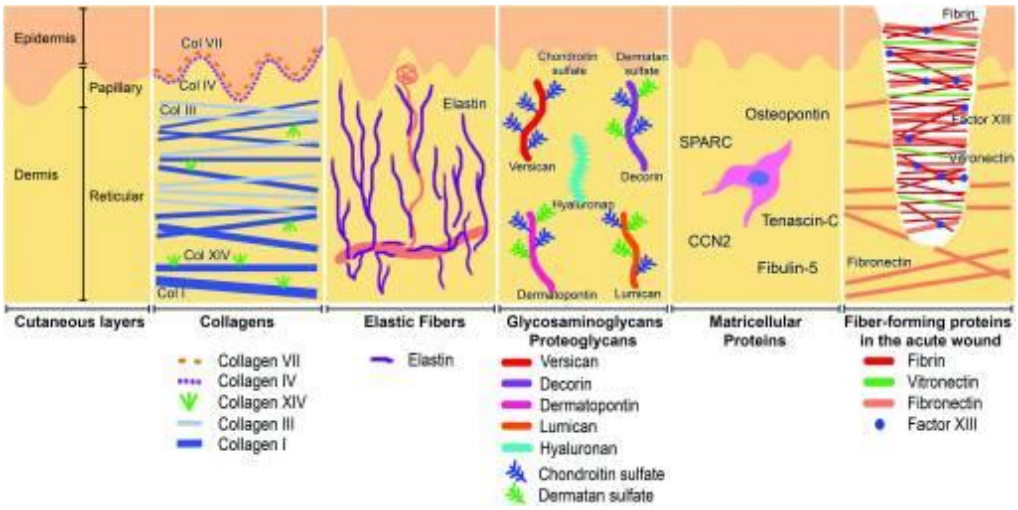


Fig. 6. ***Fiber-forming, nonfiber-forming and other selected components of ECM formation are illustrated.*** Adapted from (Tracy, Minasian et al. 2016).

4.1.3.1.2 Collagen synthesis

Collagen is an ubiquitous protein in the human body, comprising about 70% of the fat-free dry weight of human skin (Tracy, Minasian et al. 2016). It is the main structural protein in the ECM, with major tensile strength and compressive properties. Although 28 types of collagen have been identified, the most prevalent are collagen type I and III. Collagen I represents about 80-90%, and collagen III constitutes the remaining 10-20% of total collagen, while collagen V manifesting about 2% (Mariggiò, Cassano et al. 2009; Tracy, Minasian et al. 2016).

Collagen synthesis and deposition is crutial in wound healing. The predominant producer are fibroblasts. Biochemically, collagen is approximately one-third glycine such that every third amino acid is a glycine molecule, according the formula GLY-X-Y. The next most prevalent amino acid is proline (Barbul 2008; Shoulders and Raines 2009). Collagen is secreted to the extracellular space in the form of procollagen. This form is then cleaved of its terminal segments and called tropocollagen. Tropocollagen can aggregate with other tropocollagen molecules to form collagen filaments. The biosynthesis of collagen proteins includes formation of procollagen chains and subsequent proline and lysine residue hydroxylation. Hydroxyproline and hydroxylysine are essential for later glycosylation and the formation of the triple helix structure of collagen. The hydroxylation of proline and lysine residues requires oxygen, iron, and ascorbate as cofactors for successful activity. Therefore

a long-term deficiency of vitamin C results in impaired collagen synthesis (Peterkofsky 1991; Barbul 2008; Shoulders and Raines 2009). Further modifications ultimately lead to deposition of strong cross-linked collagen fibers required for proper ECM formation. A balance between collagen synthesis and breakdown is controlled by the presence of enzymes collagenases (Mariggiò, Cassano et al. 2009; Shoulders and Raines 2009).

4.1.3.2 Angiogenesis

Angiogenesis is a coordinated process, which occurs in the wound with manifestation and mitogenic stimulation of endothelial cells. The subsequent development of blood vessels is mediated through two main mechanisms, germination and cell division. The resulting vascular network is remodeled and differentiated in large and small blood vessels (Gonzalez, Costa et al. 2016).

Fibroplasia and angiogenesis are co-dependent, kind of concurrent processes, which must be successfully completed, in order to form a granulation tissue. Unbalanced regulation of any component can have significant consequences, resulting in delayed healing, chronic wounds or abnormal scar formation. Neovascularisation occurs in response to pro-angiogenic factors including VEGF, FGF, angiogenin, angiotropin, and angiopoietin 1 (Ang-1), released by infiltrating macrophages and keratinocytes (Fig. 7) (Greaves, Ashcroft et al. 2013).

The newly formed capillaries are reconstructed to transport fluid, and restore the distribution of oxygen, nutrients, immune-competent cells and other blood constituents to regenerating tissue, promoting fibroplasia and preventing sustained tissue hypoxia. Once the new blood vessels become unnecessary, disappear by apoptosis (Greaves, Ashcroft et al. 2013; Gonzalez, Costa et al. 2016).

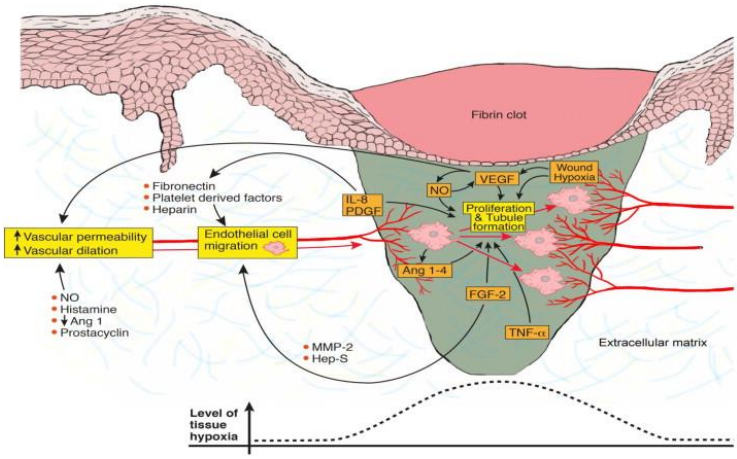


Fig. 7. ***Aspects of angiogenesis.*** Once present in the wound bed, endothelial cells proliferate and form new capillary tubules contributing to granulation tissue formation and restoration of circulatory integrity. Adapted from (Greaves, Ashcroft et al. 2013).

4.1.3.3 Epithelialization

Epithelialization is the formation of epithelium over a denuded surface, when epithelial cells migrate from the wound edges to resurface a wound defect (Beldon 2010). The reepithelialization process is arranged by basal keratinocytes at the wound edges and by epithelial stem cells from dermal appendages such as hair follicles, sweat and sebaceous glands. The process is activated by signaling pathways of cells at the wound edges, which release a plenty of different cytokines and growth factors, e.g. EGF, VEGF, keratinocyte growth factor (KGF), and insuline-like growth factor 1 (IGF-1) (Reinke and Sorg 2012).

Epithelial cells can only migrate over a moist, vascular wound surface, they are inhibited by a dry or necrotic wound (Fig. 8). A moist environment, adequate nutrition and bacterial control, lead to effective way of treatment and optimize the process of epithelialization (Enoch and Leaper 2008; Beldon 2010).

The reepithelialization process begins within hours of tissue injury. There is a marked increase in mitotic activity of the basal epithelial cells. Keratinocytes at the wound edges undergo structural changes, become flatter and elongated, that let them disociate from other epidermal cells and basement membrane. Also intracellular actin microfilaments are formed, allowing keratinocytes move across the wound surface. As the cells migrate, the overlying eschar is separated from the underlying viable tissue (Delavary, van der Veer et al. 2011; Gonzalez, Costa et al. 2016).

In order to create a path through the fibrin clot, keratinocytes in the wound edge have to dissolve the fibrin barrier. Epidermal cells secrete collagenases that break down collagen and plasminogen activators, which stimulate production of plasmin. Plasmin induces clot dissolution along the path of epithelial cell migration. Migrating epithelial cells interact with a provisional ECM (Delavary, van der Veer et al. 2011; Reinke and Sorg 2012).

Lateral migration proceeds until the defect is covered (Delavary, van der Veer et al. 2011). Further movement is halted by a contact inhibition of the cells, and a new basement membrane regenerates. Following growth and differentiation of epithelial cells constitute the new stratified epithelium (Enoch and Leaper 2008).

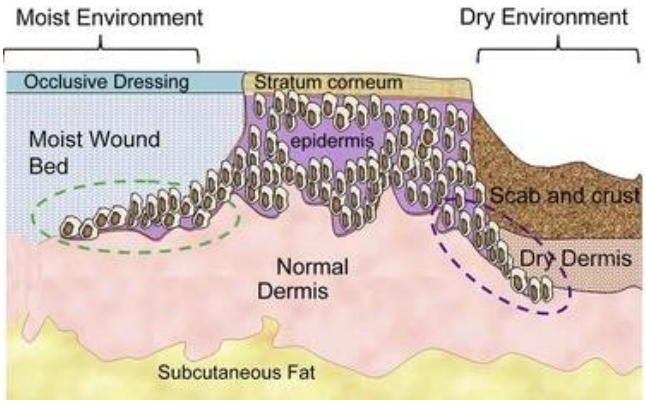


Fig. 8. ***Moist and dry environment during epithelialization.*** Adapted from (https://pocketdentistry.com/22-secondary-revision-of-soft-tissue-injury/, 18th February 2018).

4.1.3.4 Contraction

In later part of the proliferative phase, fibroblasts from the edge of the wound are stimulated by macrophages, and differentiate into myofibroblasts (Fig. 9). Myofibroblasts are contractile cells, containing α-smooth muscle actin (αSMA), commonly known as actin (Geoffrey C, Sabine et al. 2008; Greaves, Ashcroft et al. 2013). Contraction commences approximately a week after the injury, however the process is strongly dependening on activation of myofibroblasts by PDGF and TGF-β (Delavary, van der Veer et al. 2011; Greaves, Ashcroft et al. 2013).

Phenotypically, myofibroblasts are an intermediate cell type between fibroblasts and smooth muscle cells, moving closer to the edges of the wound, and becoming responsible for its contraction. Myofibroblasts are characterizased by extensive cell-matrix adhesions, abundant intercellular adherens, and they remain joined through the gap junctions. Contractility is provided by stress fibers, the bundles of actin microfilaments with non-muscle myosin. Actin microfilaments are connected by integrin receptors to the fibronectin fibrils and collagen type I and III, the components of ECM (Li and Wang 2011; Gonzalez, Costa et al. 2016). Contraction is an important part of wound healing. However, if it continues for too long, it can lead to disfigurement and loss of function (Nawaz and Bentley 2011).

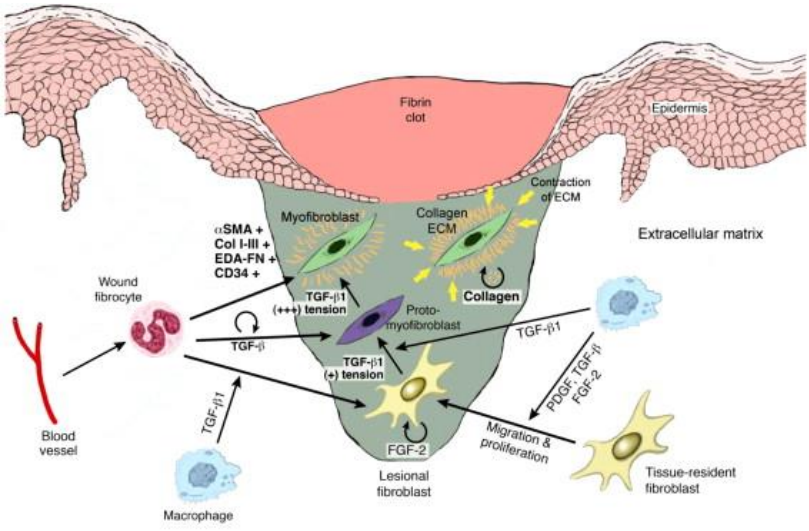


Fig. 9. ***Differentiation of fibroblasts into myofibroblasts resulting in wound contraction.*** Adapted from (Greaves, Ashcroft et al. 2013).

4.1.4 Remodeling

The remodeling phase is the last and longest phase of the wound healing. The process begins 2–3 weeks after injury and continues in order months to years. During this phase cell proliferation slows down, protein synthesis decreases, and on the contrary a formation of more organised collagen structure occurs. Most endothelial cells, macrophages and myofibroblasts undergo apoptosis, or exit the wound, leaving a mass mostly consisted of collagen and other ECM. Nutrient demand within the tissue decreases, recently formed new blood vessels regress, and the redness of the scar fade (Gurtner, Werner et al. 2008).

In addition, over 6–12 months, the acellular matrix is actively converted from proliferative type III collagen to stronger type I collagen. The process is influenced by matrix metalloproteinases (MMP), which are secreted by fibroblasts, macrophages and endothelial cells, and strengthen the repaired tissue. MMPs and their natural inhibitors are important mediators of proteolytic activity in remodeling phase. Macrophages are a rich source of MMPs and serine proteases. They are especially involved in MMP-2, MMP-12 and MMP-19 expression, and they synthesize tissue inhibitors of MMP and serine proteases. Moreover, macrophages stimulate T-cell release and differentiation to Th1 and Th2 cells, which play an important role in wound healing in case of major damage (Delavary, van der Veer et al. 2011). The wound maturation process is a balance between ECM production, tissue breakdown and remodeling. The balance is determined by among others the microenvironment, macrophage phenotype, MMP activity and T-cell response, appointing the final scar result. However, the tissue never regains the properties of uninjured skin. The scar usually achieves its maximum tensile strength by 12 weeks, with approximately 70–80% of its original strength (Geoffrey C, Sabine et al. 2008; Nawaz and Bentley 2011).

4.1.4.1 Keloid and hypertrophic scarring

A scar is an expected result of wound healing. However, in some cases, the wound healing processes may lead to excessive forms, such as keloid and hypertrophic scarring. Keloid is defined as an abnormal scar, that grows beyond the borders of the original site of tissue injury. Meanwhile hypertrophic scarring is limited to the wound margins, with a potential to

regress spontaneously. Both types cause significant amorphous growth, creating raised, red, inflexible formation of mass, responsible for serious functional and cosmetic problems. Also it can lead to symptoms such as pain or pruritus (Nawaz and Bentley 2011). Althought many pathological mechanisms, like an affected haemostasis, exaggerated inflammation, prolonged reepithelialization, overabundant ECM production, augmented neovascularization, atypical ECM remodeling or reduced apoptosis, are already known and relatively well explained, both are difficult to manage and treat. Keloid and hypertrophic scarring are very different entities. However, the pathophysiological differences between the two are still not clearly defined, and there is a need for better understanding and research (van der Veer, Bloemen et al. 2009; Nawaz and Bentley 2011).

4.2 Types of wounds

Wounds can be classified from different points of view, e.g. according to the way of origin, scale of damage, depth of tisssue injury, wound healing by primary or secondary intention, etc. However, the main perspective, crosses all of these typologies, is classification into two types of wounds – acute and chronic (Jacqui 2008; Han and Ceilley 2017).

4.2.1 Acute wound

In healthy individuals, under normal physiological conditions**,** the process of wound healing is highly efficient and the restoration of functional tissue occurs in the time frame of healing process. While a small cut is healed in a few days, major wounds may take several weeks or months to repair and result in an usually noticeable scar, depending on a size, location and other skin conditions of the wounded area (Clark, Ghosh et al. 2007; Martin and Nunan 2015).

4.2.2 Chronic wound

When the physiological repair process does not work correctly, the healing response is altered, leading to the progress of an ulcerative skin defect, a chronic wound (Martin and Nunan 2015).

There are more definitions of a chronic wound, not clearly established. One of the commonly accepted is *a wound, which is not healed in four weeks*, originally used as a standard definition of a venous leg ulcer. Another defines chronic wounds as those that fail to heal with standard therapy in an ordered and well-timed manner (Jacqui 2008). Chronic wound is also defined as an ulcer open for several weeks or more in a patient with at least one underlying comorbidity such as diabetes, obesity, vascolar disorders etc. (Snyder, Lantis et al. 2016). Anyway, there is a correlation of wound aetiologies to the time-bound healing process (Jacqui 2008; Snyder, Lantis et al. 2016).

Chronic wound is caused by two main pathways, pathobiology and microorganism invasion. That interrupts the wound healing process, heading to chronic wound expressions. Underlying pathobiology includes among others venous insufficiency, diabetes mellitus, arterial occlusion or high external pressure, leading by different mechanisms to failure of healing process. It is characterized by dysfunctional cellular events, aberrant cytokines and growth factor activity, which produce an accumulated hyperproliferative epidermal edge, creating an ulcer, that is covered with exudate and necrotic debris. Instead of a proper granulation tissue, there are vessels surrounded by fibrin cuffs, very little vessel sprouting and few or none myofibroblasts. Generally a heavy inflammatory infiltrate is produced, particularly of neutrophils, which usually differs from those in physiological healing process (Fig. 10) (Clark, Ghosh et al. 2007; Martin and Nunan 2015).

Bacteria colonizing the wound create a biofilm composed of a wide variety of polysaccharides. That protects the colonies of microorganisms, since the biofilm is relatively proof against phagocytic cells, and by the same mechanism fairly resistant to therapy as well. Frustrated phagocytes release a plenty of proteases and toxic oxygen radicals into the wound environment, making a bad situation even worse. The released agents destroy tissue cells, ECM, and growth factors in the wound. Due to that and other actions, chronic wounds absence epidermal migration and ingrowth of granulation tissue, remaining in the persisting inflammatory phase (Clark, Ghosh et al. 2007).

The majority of chronic wounds can be classified into three main categories:

- Vascular ulcers (venous and arterial ulcers) – particulary a venous leg ulcer, attributed to chronic venous insufficiency, that is caused by high pressure and congestion in veins due to thrombosis or valvular incompetence. Arterial ulcers are less common, base on arterial insufficiency primary caused by atherosclerosis, that reduces perfusion, leading to ischaemia and hypoxia.

- Pressure ulcers – is a consequence of compromised mobility and sensory perception. Systematic unrelieved pressure on the tissue restricts blood flow into the area, leading to ischaemia and reperfusion injury.

- Diabetic ulcers – based on a pathogenic triad of neuropathy, ischaemia, and trauma. Micro- and macro-circulatory dysfunctions lead to poor oxygen perfusion. Diabetic foot ulcers are a common and serious complication of diabetic patients (Frykberg and Banks 2015; Ruilong, Liang et al. 2016).

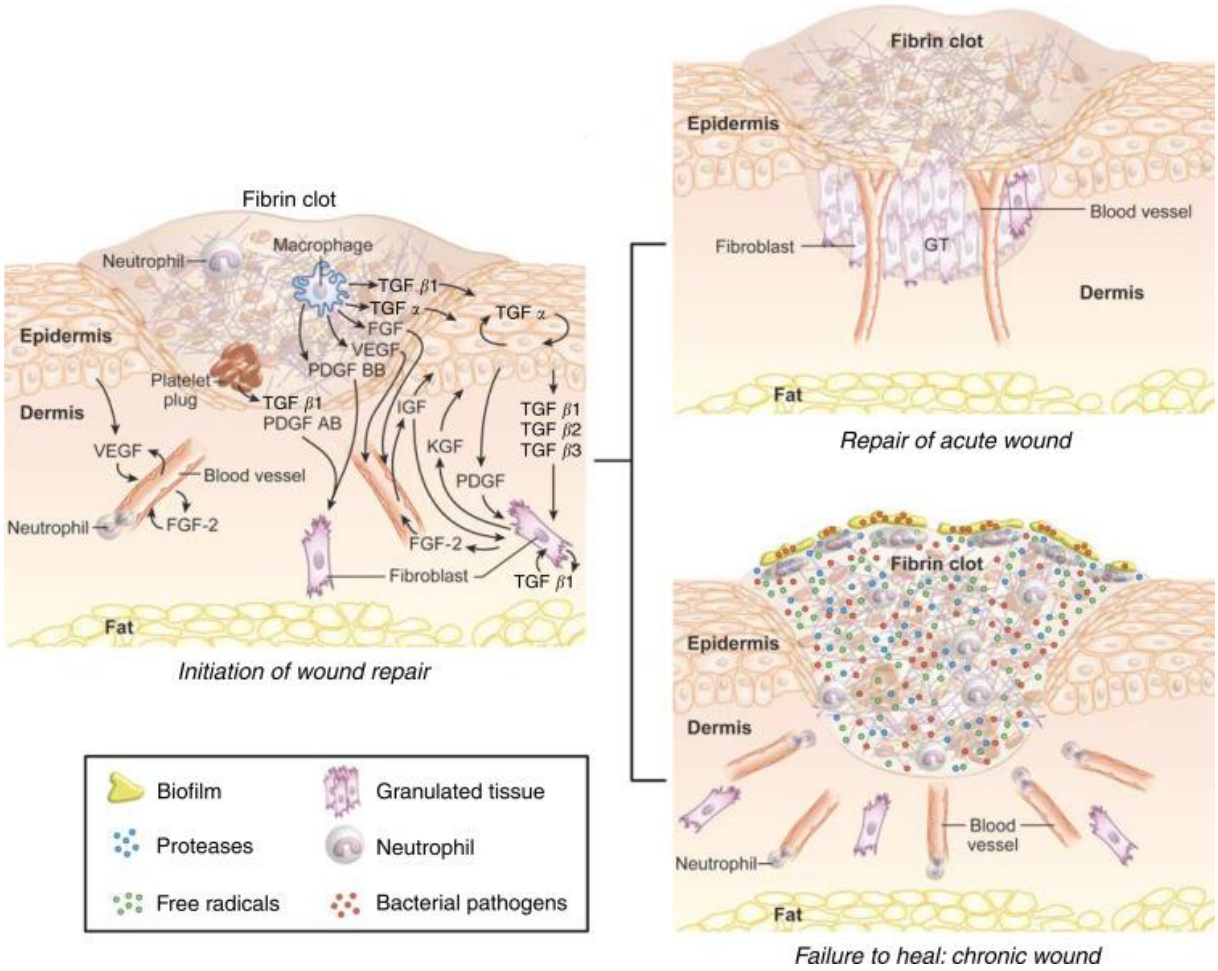


Fig. 10. ***Acute and chronic wound.*** Cellular and extracellular dysregulation affects the wound healing process. Adapted from (Clark, Ghosh et al. 2007).

4.3 Factors affecting wound healing

There are many factors that affect wound healing, which interfere with one or more phases of healing process, causing inconvenient tissue repair (Guo and DiPietro 2010). Failure of the wound healing is among others related to alterations of inflammatory response, delayed reepithelialization, improper collagen synthesis, ECM formation and remodeling, altered angiogenesis, or inadequate apoptosis (Fig. 11) (Bielefeld, Amini-Nik et al. 2013).

Factors that affect wound healing can be local or systemic. Local factors are connected to to the wound itself, meanwhile in systemic factors there is no direct relation to localization of the wound (Hajighasemali, Sadeghpour et al. 2015). Among the local factors rank oxygenation, infections, pressure, trauma, or necrosis. Cell metabolism relies on oxygen to promote wound healing and to reduce infection. When the microenvironment of wounds suffer with hypoxia, impaired vascular flow causes insufficient oxygenation, and the wound turns to chronically unhealed site. The injured skin is accesible for microorganisms causing infections, and inflammatory phase is designed to deal with it. However, if this phase is too prolonged or otherwise altered, microbial eradication would not be satisfactory, leading to failure of healing process (Guo and DiPietro 2010; Hajighasemali, Sadeghpour et al. 2015).

Systemic factors that influence healing process include age, gender, sex hormones, stress, ischemia, chronic diseases, medications, obesity, alcohol, smoking, immunodeficiency, and nutrition. Increased age is a major risk factor for delayed wound healing (Guo and DiPietro 2010). Particularly older men show delayed healing of wounds, which may be related to the role of hormones in the healing process (Soybir, Gürdal et al. 2012). Psychological stress plays also an important role in wound healing, influencing the process in several different pathways. Emotional stressors both directly (hormone and cytokine secretion) and indirectly (social behaviour) influence physiological processes and impact the healing process (Robinson, Norton et al. 2017). Chronic diseases such as diabetes mellitus have and essential drift to impaired healing, that involves hypoxia, cytokine and growth factor dysregulation, altered angiogenesis, reduced host immune responses, and neuropathy. The medications that influence wound healing are among others glucocorticoid steroids, nonsteroidal anti-inflammatory drugs (NSAIDs), or chemotherapeutics. Obesity, alcoholism, and smoking are behavioural patterns that also influence alter wound healing (Guo and DiPietro 2010). The importance of good nutrition is one of the main aspect of successful wound healing.

Carbohydrates, proteins, amino acids, fatty acids, vitamins, micronutrients, and trace elements significantly contribute to the proper wound healing process (Pollack 1979; Guo and DiPietro 2010).

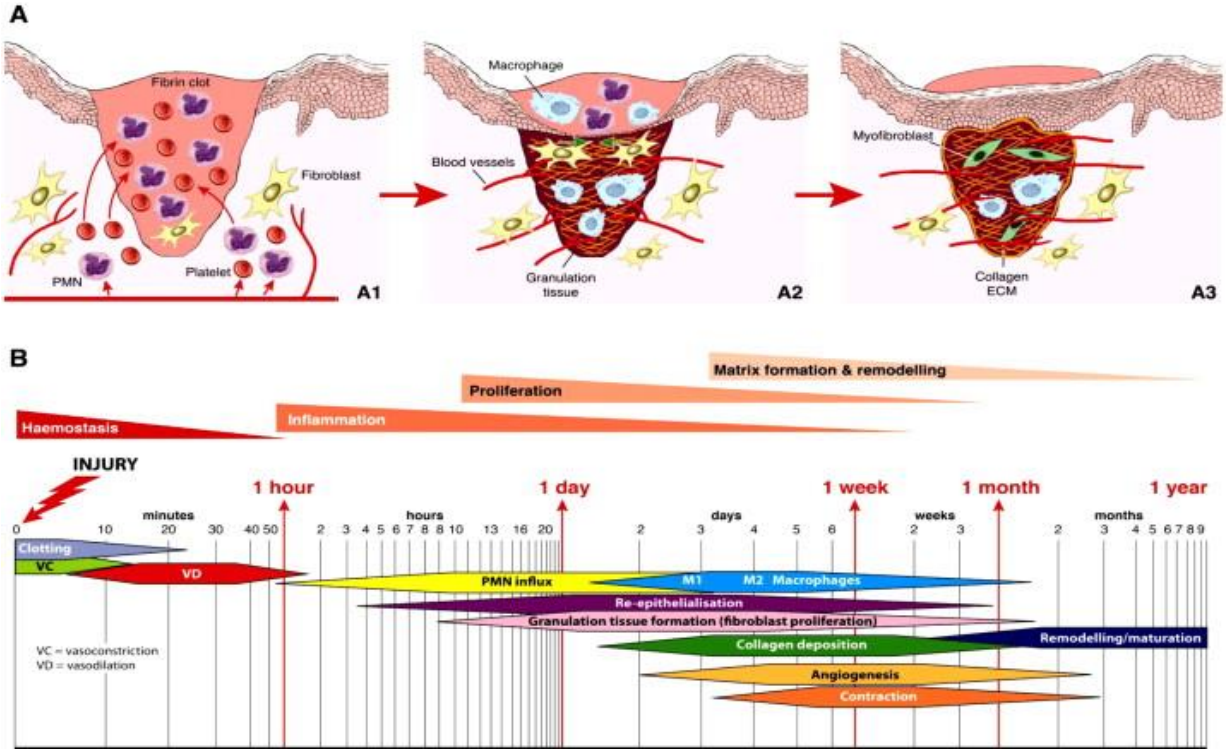


Fig. 11. ***(A) An overview of acute wound healing. (B) The time frame of four phase overlapping model in wound repair process.*** All of these courses can be influenced by local or systemic wound healing factors. Adapted from (Greaves, Ashcroft et al. 2013).

4.4 Dermal fibroblasts

Fibroblasts are one of the most common cell types, widely presented in many stuctures, particularly in connective tissue. They are a spindle-shaped heterogeneous population of cells with mesenchymal origin (Wong, McGrath et al. 2007; Li and Wang 2011). Phenotypic differencies are manifested in a variety of ways, inculding ECM production and organization, as well as the secretion of growth factors. In the skin, there are two different forms of fibroblast heterogeneity, depending on the resident depth of dermis. Fibroblasts within the deeper dermis produce less quantities of collagen than in more superficial layers (Nolte, Xu et al. 2008; Tracy, Minasian et al. 2016). Fibroblasts express an intermediate filament protein *vimentin*, a major cytoskeletal component acting as a signal integrator (Cheng, Shen et al. 2016). They are responsible for tissue homeostasis under normal physiological conditions. In injured tissues, fibroblasts are activated and differentiate into myofibroblasts, which contract and actively produce ECM proteins to facilitate wound closure (Li and Wang 2011).

4.4.1 A role of fibroblasts

Dermal fibroblasts have a crucial role in wound healing process, being the main producers of ECM, which replaces the injured tissue and forms a scaffold for tissue regeneration (Huebener and Schwabe 2013; Portou, Baker et al. 2015; Wang, Viennet et al. 2017). The process involving fibroblasts and the ECM production is known as *fibroplasia* (Greaves, Ashcroft et al. 2013). Wound fibroblasts located in the dermis originate from tissue-resident mesenchymal cells, circulating fibrocytes, or bone marrow-derived precursor cells (Spyrou, Watt et al. 1998; Shaw and Martin 2009; Barisic-Dujmovic, Boban et al. 2010; Huebener and Schwabe 2013).

The main processes involving fibroblasts leading to ECM formation include fibroblast migration, proliferation, phenotype differentiation and collagen synthesis. These actions arise under the influence of growth factors such as fibronectin, PDGF, FGF, TGF-β and IGF-1, which are mainly produced by macrophages (Delavary, van der Veer et al. 2011; Portou, Baker et al. 2015). On the contrary fibroblasts produce a plethora of cytokines, chemokines

and growth factors as well, affecting via other cell types essential wound healing processes as a granulation tissue formation, angiogenesis, reepithelialization and wound contraction (Fig. 12) (Greaves, Ashcroft et al. 2013; Li, Li et al. 2017).

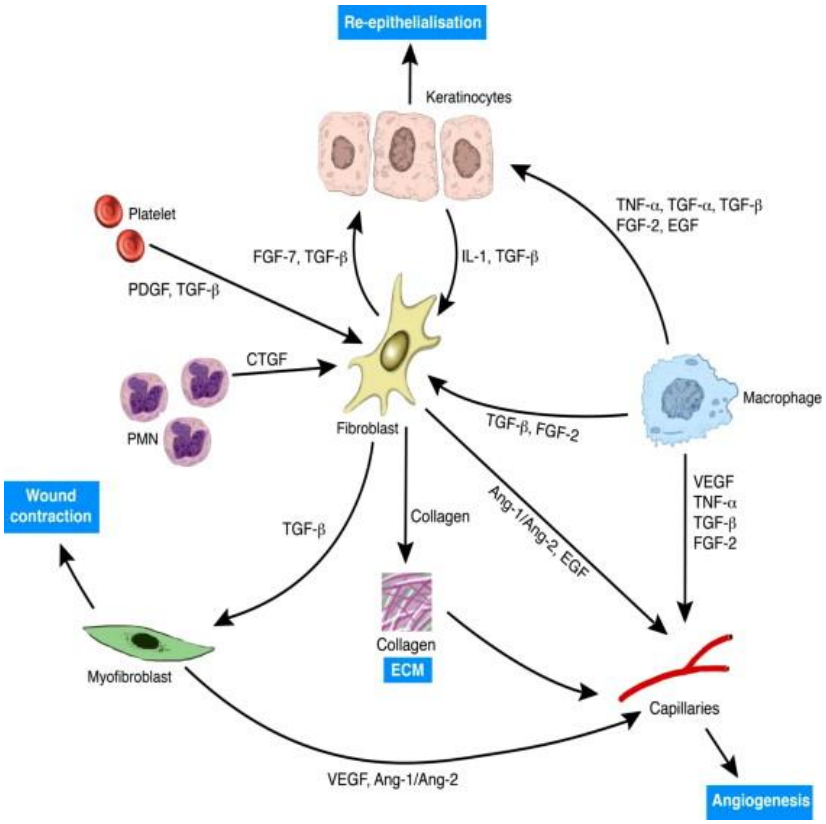


Fig. 12. ***Interaction of fibroblasts with various cell types and cytokines in the wound bed, leading to complex wound repair.*** Adapted from (Greaves, Ashcroft et al. 2013).

Activated fibroblasts produce collagen and other ECM components, gradually replacing provisional fibrin matrix. Collagen synthesis is initiated between 3 and 5 days after an injury (Delavary, van der Veer et al. 2011; Bhattacharyya, Kelley et al. 2013; Wang, Viennet et al. 2017). The new ECM is primarily composed of collagen, glycosaminoglycans, proteoglycans, fibronectin and elastin. Particularly TGF-β and PDGF grow factors are responsible for the ECM production and deposition (Delavary, van der Veer et al. 2011; Portou, Baker et al. 2015).

Later in the proliferative phase fibroblasts convert to proto-myofibroblasts in response to increased tissue tension and TGF-β expression. A positive feedback loop of tension and TGF-β release lead to final maturation of myofibroblasts, which generate the majority of contractile

forces in the wound. Myofibroblasts are characterized by increased expression of αSMA, collagen type I and III, vimentin, desmin, and myosin (Li and Wang 2011). Compared to fibroblasts, myofibroblasts produce higher amounts of ECM components (Delavary, van der Veer et al. 2011; Greaves, Ashcroft et al. 2013).

Fibroblasts have been found to express the full range of human Toll-like receptors (TLRs) from 1 to 10 (Portou, Baker et al. 2015). Activation of TLR-4 results in elevated collagen synthesis and increased expression of multiple genes involved in tissue remodeling and ECM production. Moreover, TLR-4 dramatically enhances the sensitivity of fibroblasts to the stimulatory effect of TGF-β (Bhattacharyya, Kelley et al. 2013).

TGF-β is one of the most important agent throughout wound healing courses especially in proliferative phase. TGF-β stimulates fibroblast migration and proliferation, it promotes MMPs expression to anable fibroblasts overcoming cellular debris, and modulates collagen production. Together with VEGF contributes to neoangiogenesis. Furthermore, TGF-β plays a key role in reepithelialization and phenotypic differentiation (Mariggiò, Cassano et al. 2009). TGF-β has three isotypes (TGF-β1, -β2 and -β3), which all stimulate infiltration of inflammatory cells and fibroblasts. However, fetal skin is associated with scarless repair, when low levels of TGF-β1 and high levels of TGF-β3 are expressed. That suggests each subtype may be a crucial factor in scarring process (Delavary, van der Veer et al. 2011; Bhattacharyya, Kelley et al. 2013). TGF-β releasing is mediated particularly by macrophages and platelets (Mariggiò, Cassano et al. 2009).

Macrophages have an eminent position in wound healing process, provide a source of grow factors and pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6 and TNF-α, which are responsible for the control of inflammatory cell adhesion, migration, and proliferation. Ablation of macrophages in the wound consequently results in decreased expression of TGF-β, reduced fibroblast proliferation and diminished ECM deposition (Delavary, van der Veer et al. 2011).

Fibroblasts are among others involved in fibrinolysis through secretion of u-PA and t-PA to enable later tissue remodeling and migration across the ECM. They catabolically modulate ECM via up-regulated u-PA, t-PA, MMP-1, and MMP-3, while down-regulating profibrotic CTGF, collagen I, collagen III, fibronectin, PAI-1, TIMP-2,3 and α-SMA (Greaves, Ashcroft et al. 2013). Increasing quantities of ECM signal fibroblasts to decrease subsequent collagen

production. Furthermore, IFN-γ and TNF-α stimulate fibroblasts to decrease collagen synthesis. Fibroblasts stop producing collagen and former granulation tissue is replaced by a relatively acellular scar formation (Delavary, van der Veer et al. 2011; Greaves, Ashcroft et al. 2013)

4.4.2 Migration

Fibroblast migration is a complex process in skin wound healing, which requires the coordination of various growth factors and cytokines. Particularly macrophage-derived PDGF, TGF-β and FGF-2 result in cell migration from surrounding healthy tissue to the wound site (Greaves, Ashcroft et al. 2013; Zhu, Sun et al. 2017). Process of migration is characterized via several particular actions, including lamellipodium extension at the front edge of cell, expression of adhesive receptors, secretion of surface proteases leading to proteolysis, and contraction by actomyosin complexes (Fig. 13) (Parri and Chiarugi 2010).

Growth factors express in fibroblasts specific integrin transmembrane receptors, heterodimers with α and β chains, facilitating particularly cell adhesion. Integrins perform both inside-out and outside-in signaling, altering their binding affinity for ligands during the time. Initially α3β1 and α5β1 integrin subunits are expressed, which enable binding to noncollagen ECM proteins. Later collagen deposition results in increased α2β1 subunit, recognized as the fibroblast-collagen binding integrin. This and other subunit changes facilitate efficient fibroblast migration through a provisional ECM into the wound space (Li, Fan et al. 2004; Greaves, Ashcroft et al. 2013; Tracy, Minasian et al. 2016).

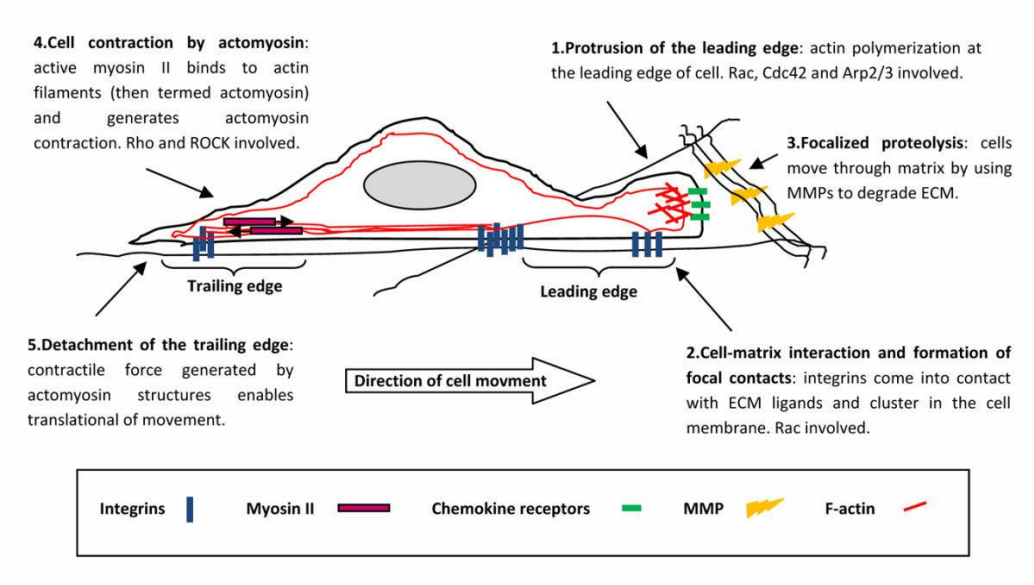


Fig. 13. ***Cell migration is a multistep process involving changes in the cytoskeleton, cell-substrate adhesions and ECM components.*** Adapted from (Parri and Chiarugi 2010).

Migrating fibroblasts are front-rear polarized cells with enlongated-shape enable the movement in one direction (Parri and Chiarugi 2010). Cell polarization occur through activation of the small Rho GTPases, which in turn organizes dynamic actin polymerization and lamellipodium formation. There are three main members of Rho family GTPases, Rho, Rac and Cdc42. Rac is primarily responsible for lamellipodium formation at the leading edge, Cdc42 mainly affects cell polarity and filopodia protrusions, and Rho influences in particular actomyosin contraction (Ridley 2015). An actin cytoskeleton is one of the major factor required for cell migration. Movement of the cell body is dependent on contractility generated by actin and myosin filaments. Some focal complexes develop into large adhesions enable actomyosin contractile forces. The role of Rho GTPases and its effector Rho-associated protein kinase (ROCK) is intricate. For cell migration their activity needs to be reduced in protrusions at the front of the cell, but keeping the retraction in the trailing edge. That allows the cells to extend the leading edge and attach to the surface, while the rear edge contract and push the cell forward (Franz, Jones et al. 2002; Parri and Chiarugi 2010; Trepat, Chen et al. 2012). Furthermore, Rho proteins regulate several other processes relevant to cell migration, such as cell-substrate adhesion, cell-cell adhesion, protein secretion, or vesicle transcription (Parri and Chiarugi 2010). Focal adhesion kinase (FAK) is an expressive agent in cell movement. FAK is required for the signaling cascade initiated by the interaction between integrins and ECM proteins. FAK-deficient fibroblasts show significantly

decreased cell migration (Zhao, Cheng et al. 2016). Furthermore, fibronectin is one of the most important ECM glycoproteins, promoting the fibroblasts adhesion and migration through the ECM (Mariggiò, Cassano et al. 2009). The process is also dependent on degradation of the ECM via proteolysis mediated by proteases, particularly MMPs and u-PA (Parri and Chiarugi 2010).

Fibroblast migration into wounded area is one of the initial action with impact on the consequence of wound healing and tissue formation. The speed and efficiency of migration is affected by many factors including the microtubule network, expression levels of adhesion receptors, and secretion of proteases, that degrade ECM proteins and create the path. However, persistent fibroblast migration results in excessive tissue remodeling, usually leads to a fibrotic scar formation (Franz, Jones et al. 2002; Zhao, Cheng et al. 2016).

4.4.3 Proliferation

Once fibroblasts are attracted into the wound space, the process of *fibroplasia* can be initiated (Greaves, Ashcroft et al. 2013). Fibroblast proliferation and deposition of specific ECM components, followed by wound contraction and remodeling, are required for proper wound closure (Delavary, van der Veer et al. 2011). Among molecular mechanisms responsible for the proliferative effects belong activation of *mitogen-activated protein kinase* (MAPK) represent important mediators of signal transduction pathways, facilitate the effects of growth factors and other proteins. At least 3 MAPK families have been characterized: *Extracellular signal-regulated kinases* (ERK), *Jun kinase* (JNK), and *p38*. MAPKs, particularly ERK, play an important role in proliferation, differentiation, and survival processes. Phosphorylation of ERK is connected with activation of CREB (cAMP response element-binding protein). Phosphorylated ERK translocates to the nucleus, where phosphoryling CREB, a transcription factor, which activates genes involved in cell proliferation. Furthermore, anti-apoptotic *Phosphoinositide 3-kinase* PI3K/AKT is also an important signaling pathway for cell survival other than MAPK signaling (Fig. 14) (Fujiwara, Kanazawa et al. 2014).

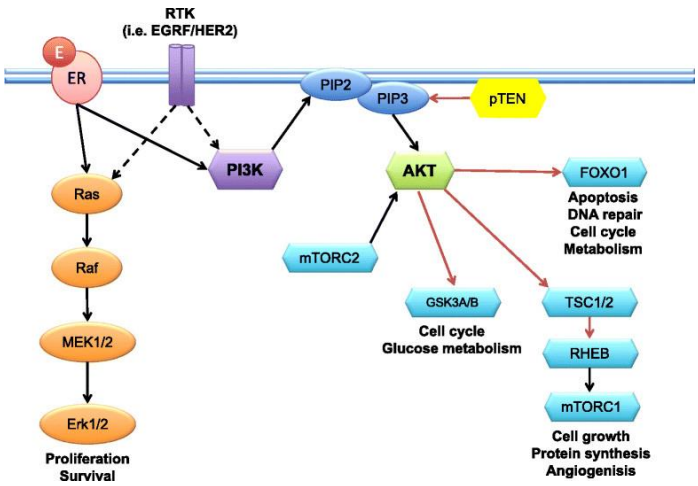


Fig. 14. ***The PI3K/AKT and MAPK (also known as RAS/RAF/MEK/ERK) signaling pathways.*** P*rotein kinase B* known as AKT activates several enzymes and transcription factors, including mammalian target of rapamycin (mTOR). The MAPK pathway is an alternative way of mTOR activation. Adapted from (Toss and Cristofanilli 2015). RTK, Receptor tyrosine kinases; ER, Estrogen receptor

Protein vimentin is required to support ERK-mediated phosphorylation and activation. Vimentin serves as an integrator of the processes that occur during wound healing, regulating the fibroblast proliferation and epithelial–mesenchymal transition (EMT), both crucial processes for successful wound repair. In the absence of vimentin, these cellular processes are inhibited, thereby disrupting normal tissue regeneration, reepithelialization, and the formation of a scar (Cheng, Shen et al. 2016).

TGF-β is a crutial cytokine in both fibroblast migration and proliferation. Particularly through this grow factor integrin expression is stimulated, facilitating the cell migration into the wound site. TGF-β and other mediators influence many signaling cascades, including Ras/MAPK, PI3K/AKT/mTOR, Rho GTPase, Cyclin-dependent kinases (CDKs), Wnt and Hedgehog signaling pathways (Fig. 15). All of that contributes to regulation of cell growth, survival, and differentiation (Carels, Spinassé et al. 2016; Cheng, Shen et al. 2016; Tracy, Minasian et al. 2016; Zhu, Sun et al. 2017).

Generally cell proliferation is required to replace cells that have been lost as a result of injury or cell death. Thus the process of proliferation is a fragile balance with cell death to maintain an adequate number of cells in the injured tissue. During the proliferative phase of

wound healing, a granulation tissue, composed of new blood vessels, fibroblasts and other ECM components, is formed (Nolte, Xu et al. 2008; Carels, Spinassé et al. 2016).

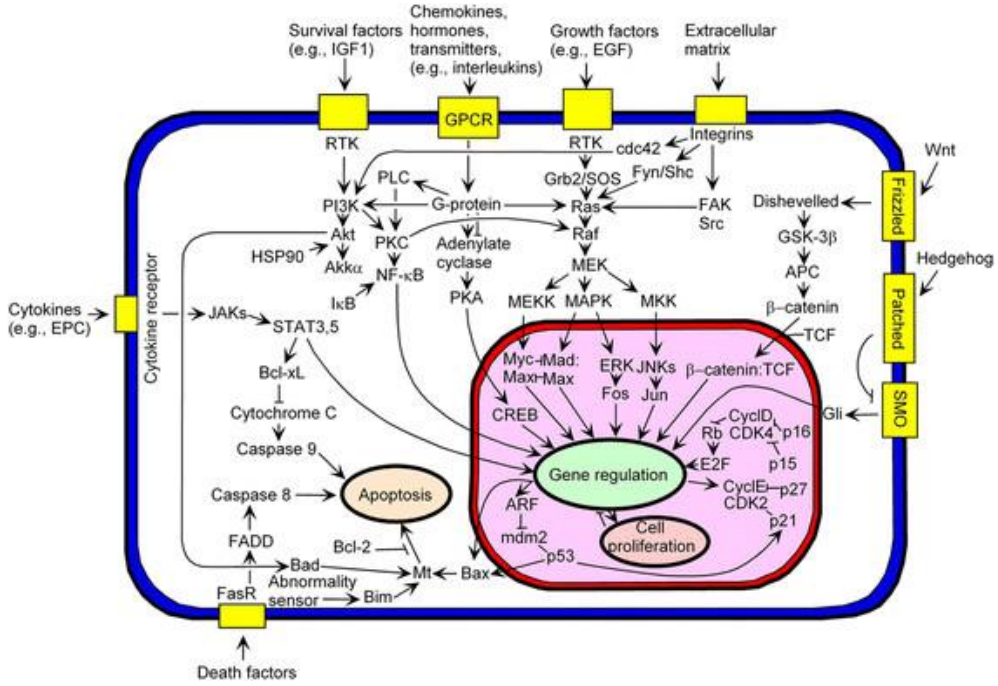


Fig. 15. ***Map of key proteins and mediators in signaling pathways affecting cell migration, proliferation, differentiation and other aspects of cell survival or death.*** Adapted from (Carels, Spinassé et al. 2016).

5 Methods and materials

5.1 Plant extracts

Plants were collected on Samoa Islands (the Independent State of Samoa) in the South Pacific Ocean (Coleman 2015) by a research group under the leading of Prof. Kokoška (Czech University of Life Sciences Prague, Faculty of Tropical Agro Sciences) in September 2015. All plant species were selected on the basis of traditional use by indigenous people to heal wounds. The plant extracts were prepared by maceration in 80% ethanol. Dry residues were dissolved in dimethyl sulfoxide (DMSO) at a concetration 51,2 mg/ml as stock solutions (Czech University of Life Sciences Prague, Faculty of Tropical Agro Sciences) and stored at -20°C until to use.

5.1.1 Classification

The nominal list of tested extracts is referred in Table 3. 16 extracts from 14 plant species were examined. In the case of selected plants (*Inocarpus fagifer, Premna serratifolia)* were used two different parts of the plant (leaves and bark) as seperated samples. The other tested plants were run with one extract sample.

Table 3. ***Tested plant extracts.*** Preparation and classification were performed under the supervision of Prof. Kokoška (Czech University of Life Sciences Prague, Faculty of Tropical Agro Sciences).

Species	***Local name***	***Family***	***Used part***	***Evidence ID***
Barringtonia asiatica	*Futu*	*Barringtoniaceae*	*Seed*	*BA*
Cerbera manghas	*Leva*	*Apocynaceae*	*Leaves*	*CM*
Commelina diffusa	*Mauutoga*	*Commellinaceae*	*leaves + vining stem*	*CD*
Inocarpus fagifer	*Ifi*	*Fabaceae*	*Leaves*	*IT*
Inocarpus fagifer	*Ifi*	*Fabaceae*	*Bark*	*IFag*
Kleinhovia hospita	*Fuafua*	*Sterculiaceae*	*Bark*	*KH*
Mikania micrantha	*Fue saina*	*Asteraceae*	*leaves + vining stem*	*MM*
Omalanthus nutans	*Mamala, Fanuamamala*	*Euphorbiaceae*	*leaves + vining stem*	*ON*
Peperomia pellucida	*vao vai*	*Piperaceae*	*Leaves*	*PP*
Phymatosorus scolopendria	*Lau maga maga*	*Polypodiaceae*	*leaves + vining stem*	*PhS*
Piper graeffei	*Fue mangoi*	*Piperaceae*	*leaves + little among of vining stem*	*PG*
Premna serratifolia	*Aloalo*	*Verbenaceae*	*Leaves*	*PS*
Premna serratifolia	*Aloalo*	*Verbenaceae*	*Bark*	*PrS*
Psychotria insularum	*Matalafi*	*Rubiaceae*	*Leaves*	*PI*
Schizostachyum glaucifolium	*Ofe*	*Poaceae*	*Leaves*	*SG*
Solenostemon scutellarioides	*Pate*	*Lamiaceae*	*Leaves*	*SS*

5.2 *In vitro* cell culture

Normal human dermal fibroblasts (NHDF) were isolated from the facial skin removed during cosmetic plastic surgery with an informed agreements of the donors. NHDF were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific), 1mM L-glutamine (Gibco, ThermoFisher Scientific), 1% nonessential amino acids (Biosera), penicillin (100 U/ml) and streptomycin (0,1 mg/ml) (Biosera), and incubated in 5% CO_2 at 37°C. Cell passages between 2 and 9 were used throughout the experiments.

5.3 Cell Proliferation Assay

NHDF were seeded on 96-well plates (Techno Plasstic Products, Switzerland) at a density of cells 9 000/cm^2 in 150 µl of medium per well and incubated at 5% CO_2 and 37°C over the night. Medium was replaced with 150 µl of serial dilutions of 16 extracts (512, 256, 128 and 32 µg/ml), respectively serial dilutions of 3 selected extracts (16, 8, 4, 1 µg/ml) and incubated for 24, 48 and 72 hours. Along the samples, controls (nontreated cells) at the time points 0, 24, 48 and 72 hours, and blanks (no cells) for media background fluorescence measurement, were evaluated. After treatment, medium was removed and all samples were washed out with phosphate buffered saline (PBS) twice. Each microplate was stored dry at −80°C until all time points had been collected. During freezing step complete lysis of the cells was achieved. All microplates were then thawed at room temperature, and 100 µl of the fluorescent dye in cell lysis buffer (Invitrogen, ThermoFisher Scientific) was added to each sample well. The samples were incubated in darkness for 2-5 min, and then the fluorescence was measured by microplate reader (Biotek Synergy HT) in black 96 well plates at ex 485nm/em 528nm according to the manufacturer's instructions. Experiments were performed on medium supplemented with 10% FBS and serum free medium. All experiments were repeated at least three times.

5.4 Scratch Wound Assay

NHDF were seeded on 6-well plates (Techno Plasstic Products, Switzerland) at a density of cells 23 000/cm^2 and cultivated at 37°C and 5% CO_2 until 90% confluence was reached. Then the cell monolayers were scratched by 10 µl pipette tip to create four crosses (open area) per well (Fig. 16).

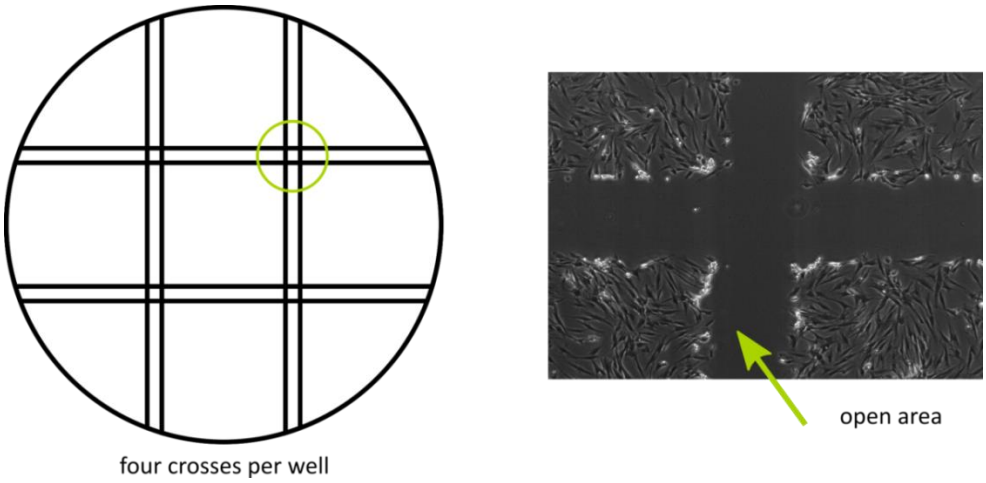


Fig. 16. ***Illustration of scratch wound assay into the dermal fibroblast monolayer.***

After the wounding, medium was removed and the cells were treated with 2 ml of serial dilutions of 16 extracts at a concentration 32 µg/ml, respectively serial dilutions of 3 selected extracts (16, 8, 4, 1 µg/ml). Cell migration was monitored and photographed by phase contrast microscopy (Olympus IX83) using 4x magnification objective immediately after the treatment (0h) and every 24 hours for 3 days (24h, 48h, 72h). Final images were analysed by TScratch software (CSELab, ETH Zurich). The software detected area covered by cells and evaluated an area without cells with an expression to the whole picture (open area with no cells = 1, area completely closed by cells = 0). Percentage of the open area was assessed and compared to the control treatment. The reduction of open area indicated the migration of cells. All treatments were performed in a serum free medium to eliminate cell proliferation and the serum free medium was applied as a control during all experiments. All experiments were run on 3 to 6 measurements.

5.5 Statistics

All data are reported as a mean ± standard error of mean (SEM). The data normality was tested by Shapiro-Wilk test, and for comparison treated-untreated groups Wilcoxon match paired test was performed. The differences were considered statistically significant with values $p \leq 0,05$. All statistical evaluations were carried out with Statistica Version 10 software (StatSoft, USA).

6 Results

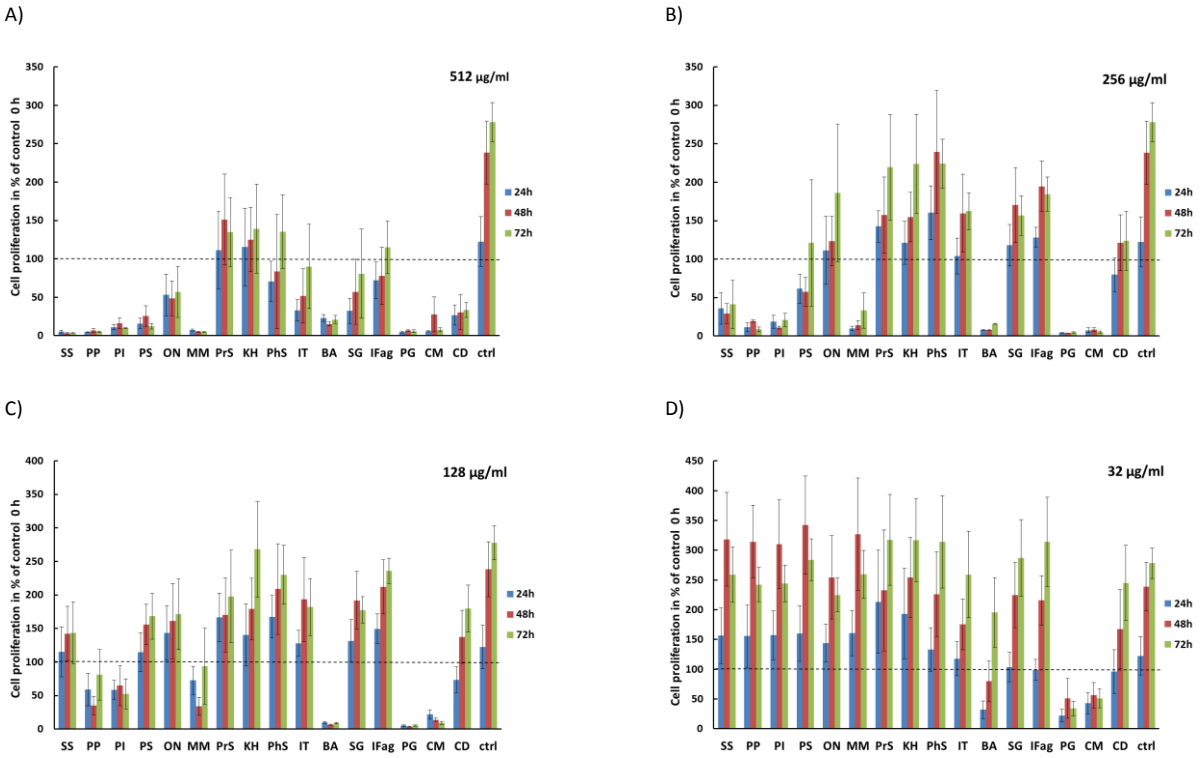
6.1 Screening of all extracts

6.1.1 The effect of extracts on NHDF proliferation

Screening was performed with all 16 extracts at concentrations 512, 256, 128 and 32 µg/ml. Proliferation of cells was evaluated by DNA quantification using PicoGreen dye, that strongly fluoresces when bound to nucleic acids. The experiments were done in medium supplemented with FBS and serum free medium.

6.1.1.1 Medium with FBS

NHDF were cultured in medium supplemented with 10% FBS and treated with 16 plant extracts: BA, CD, CM, IFag, IT, KH, MM, ON, PG, PI, PP, PS, PhS, PrS, SG, SS at concentrations 512, 256, 128 and 32 µg/ml for 72 hours. Every 24 hours the cell proliferation was measured. Results of all extracts on NHDF proliferation at various concentrations are summarized in Graph 1.



Graph 1. ***The effect of extracts on NHDF proliferation in medium supplemented with 10% FBS.*** NHDF were treated with 16 extracts (BA, CD, CM, IFag, IT, KH, MM, ON, PG, PI, PP, PS, PhS, PrS, SG, SS) at concentrations A) 512 µg/ml, B) 256 µg/ml, C) 128 µg/ml and D) 32 µg/ml. The cell proliferation was measured by DNA quantification in time points 24, 48, 72 h, and was expressed as percentage of the untreated control in time 0 h (dashed line). As a control during the experiment served untreated NHDF cells (ctrl). The values are represented as a mean ± SEM from 3 independent experiments.

Screening of all extracts was evaluated in three time points 24, 48, 72 h and compared to untreated control in time 0 h. The results showed various effect of extracts on NHDF proliferation with a concentration dependence. While at the concentration 512 µg/ml were cytotoxic (decreased proliferation more than 70%) 8 extracts, at the concentration 32 µg/ml expressively reduced cell proliferation only 3 extracts. Generally, extracts at the concentration 32 µg/ml referred the highest values. Interestingly, contra to the concentration dependence, there is no time correlation in all the cases. Certain extracts manifested increased cell proliferation in time 48 h than 72 h, particularly at the concetration 32 µg/ml.

At the concetration 512 µg/ml 8 extracts (SS, PP, PI, PS, MM, PG, CM, CD) decreased proliferation by more than 70% in all the time points. 3 extracts (ON, IT, SG) reduced cell proliferation by less than 50% and 4 extracts (Pr, KH, PhS, IFag) slightly stimulated cell proliferation compared to the untreated control 0 h particularly in the time point 72 h.

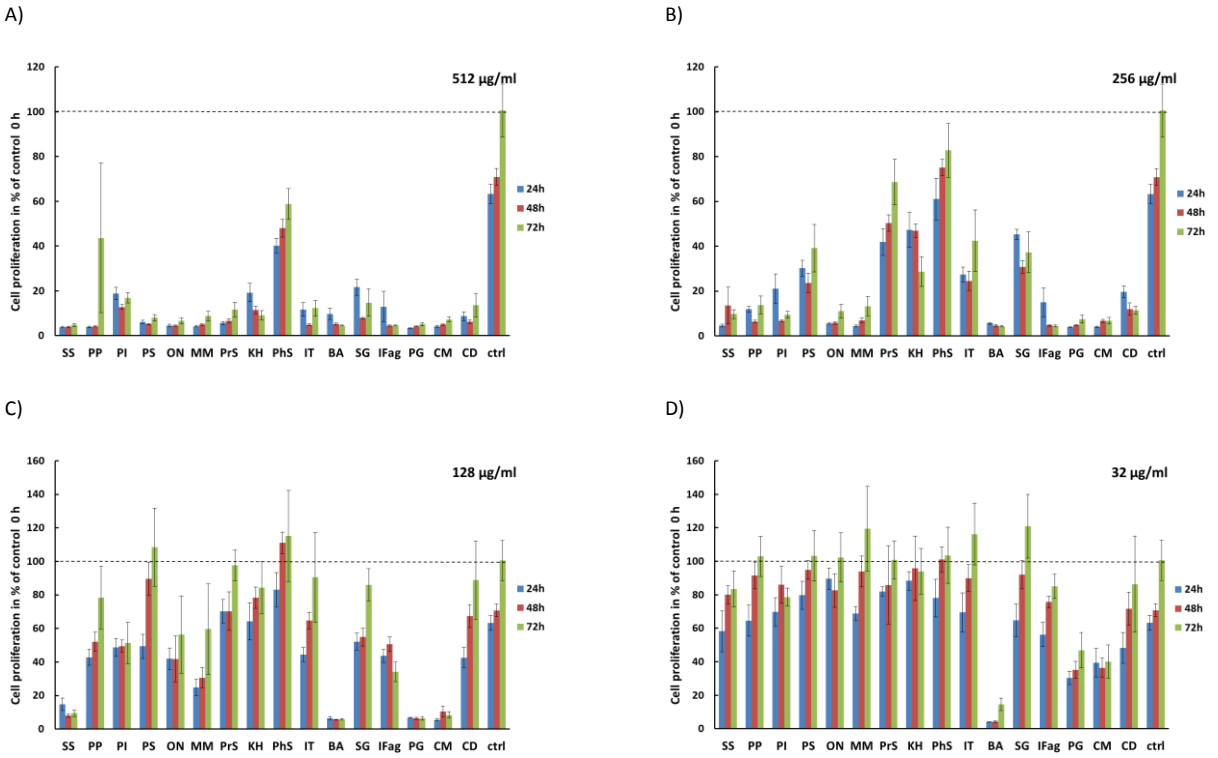
At the concentration 256 µg/ml none of extracts decreased cell proliferation by more than 70%. 7 extracts (SS, PP, PI, MM, BA, PG, CM) inhibited NHDF proliferation more than 50%, 2 extracts (PS, CD) reduced proliferation less than 50%, and the rest of extracts (ON, PrS, KH, PhS, IT, SG, IFag) had no cytotoxic effect or increased proliferation in all the time points compared to the untreated control 0 h.

At the concentration 128 µg/ml 3 extracts (BA, PG, CM) decreased NHDF proliferation more than 70%. 3 extracts (PP, PI, MM) oscillated under the control level in all the time points, and the other 10 extracts (SS, PS, ON, PrS, KH, PhS, IT, SG, IFag, CD) supported cell proliferation in comparison to the control 0 h.

In the case of concentration 32 µg/ml NHDF proliferation was decreased more than 40% by extracts PG, CM and partially by extract BA. The remaining 13 extracts (SS, PS, PP, PI, MM, ON, PrS, KH, PhS, IT, SG, IFag, CD) significantly increased NHDF proliferation, particularly in the time points 48 and 72 h. Suprisingly, 6 extracts (SS, PS, PP, PI, MM, ON) reported distinctly increased effect on NHDF proliferation in time 48 h than 72 h. The best results showed extracts PrS, KH, PhS and IFag, that after 72 h of treatment referred more than 3 times increased proliferation versus untreated control 0 h.

6.1.1.2 Serum free medium

NHDF were cultured in serum free medium and affected by extracts BA, CD, CM, IFag, IT, KH, MM, ON, PG, PI, PP, PS, PhS, PrS, SG, SS at concentrations 512, 256, 128 and 32 µg/ml for 72 hours as in the case of medium with FBS. Results are sumarized in Graph 2.



Graph 2. ***The effect of extracts on NHDF proliferation in serum free medium.*** NHDF were treated with 16 extracts (BA, CD, CM, IFag, IT, KH, MM, ON, PG, PI, PP, PS, PhS, PrS, SG, SS) at concentrations A) 512 µg/ml, B) 256 µg/ml, C) 128 µg/ml and D) 32 µg/ml. The cell proliferation was measured by DNA quantification in time points 24, 48, 72 h and was expressed as percentage of the untreated control in time 0 h (dashed line). As a control during the experiment served untreated NHDF cells (ctrl). The values represent a mean ± SEM from 3 independent experiments.

Screening of the extracts in serum free medium showed different results compared to NHDF cultured in medium with 10% FBS. Number of cells increased significantly slower without FBS. However, the principle of positive or negative effect of the extracts on NHDF proliferation and concentration dependence, were preserved.

At the concetration 512 µg/ml an expressive reduction of NHDF proliferation in the cases of all extracts was observed. 15 extracts decreased NHDF proliferation more than 70%, extract PhS showed reduction about 50% to the untreated control 0 h.

At the concentration 256 µg/ml 10 extracts (SS, PP, PI, ON, MM, BA, IFag, PG, CM, CD) decreased proliferation more than 70%, and 6 extracts (IT, KH, PS, PhS, PrS, SG) reduced proliferation variously (20-60%) under the control level 0 h.

In the case of concentration 128 µg/ml 4 extracts (SS, BA, PG, CM) reduced cell proliferation more than 70%. The other extracts oscillated under the control level, meanwhile

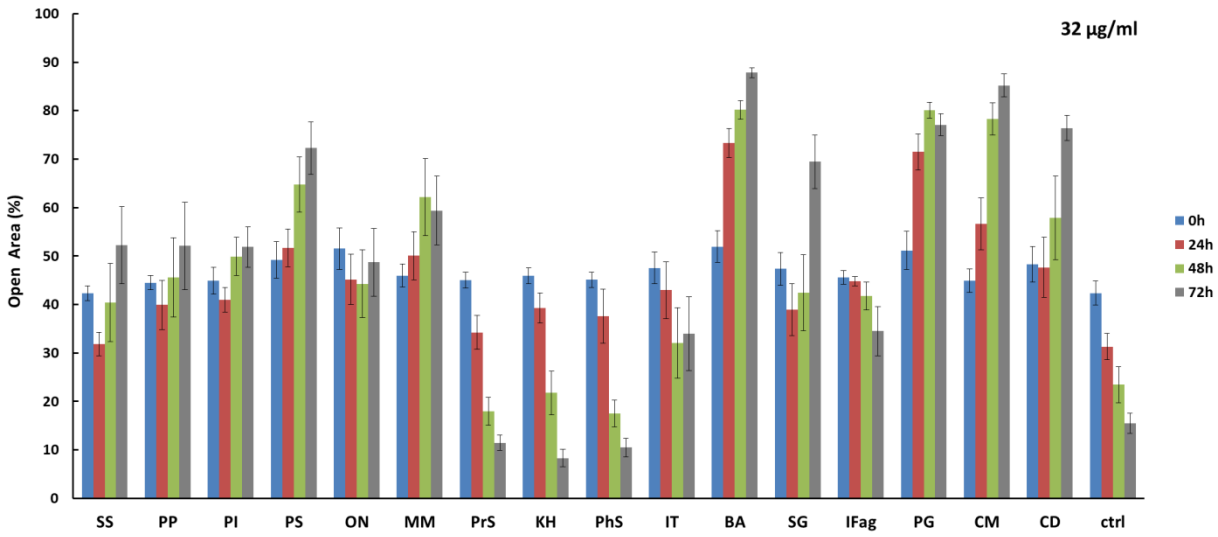
2 extracts (PS, PhS) reported increase of cell proliferation particularly in the time point 72 h compared to the control 0 h.

At the concentration 32 μg/ml distinctly decreased cell proliferation extract BA (more than 70%), PG and CM (more than 50%). 4 extracts demonstrated after 72 h of treatment reduction of cell proliferation less than 25%. The other extracts (PP, PS, ON, MM, PrS, KH, PhS, IT and SG) showed similar or increased levels of NHDF proliferation versus control 0 h.

6.1.2 The effect of extracts on NHDF migration

NHDF migration was evaluated by scratch wound assay and monitored every 24 hours for 3 days. Scratch wound assay was done with all extracts at concentration of 32 μg/ml. Higher concetrations were cytotoxic or decreased cell proliferation significantly in majority of cases. Scratch wound assay was run on serum free medium to avoid effect of cell proliferation (see chapter 6.1.1).

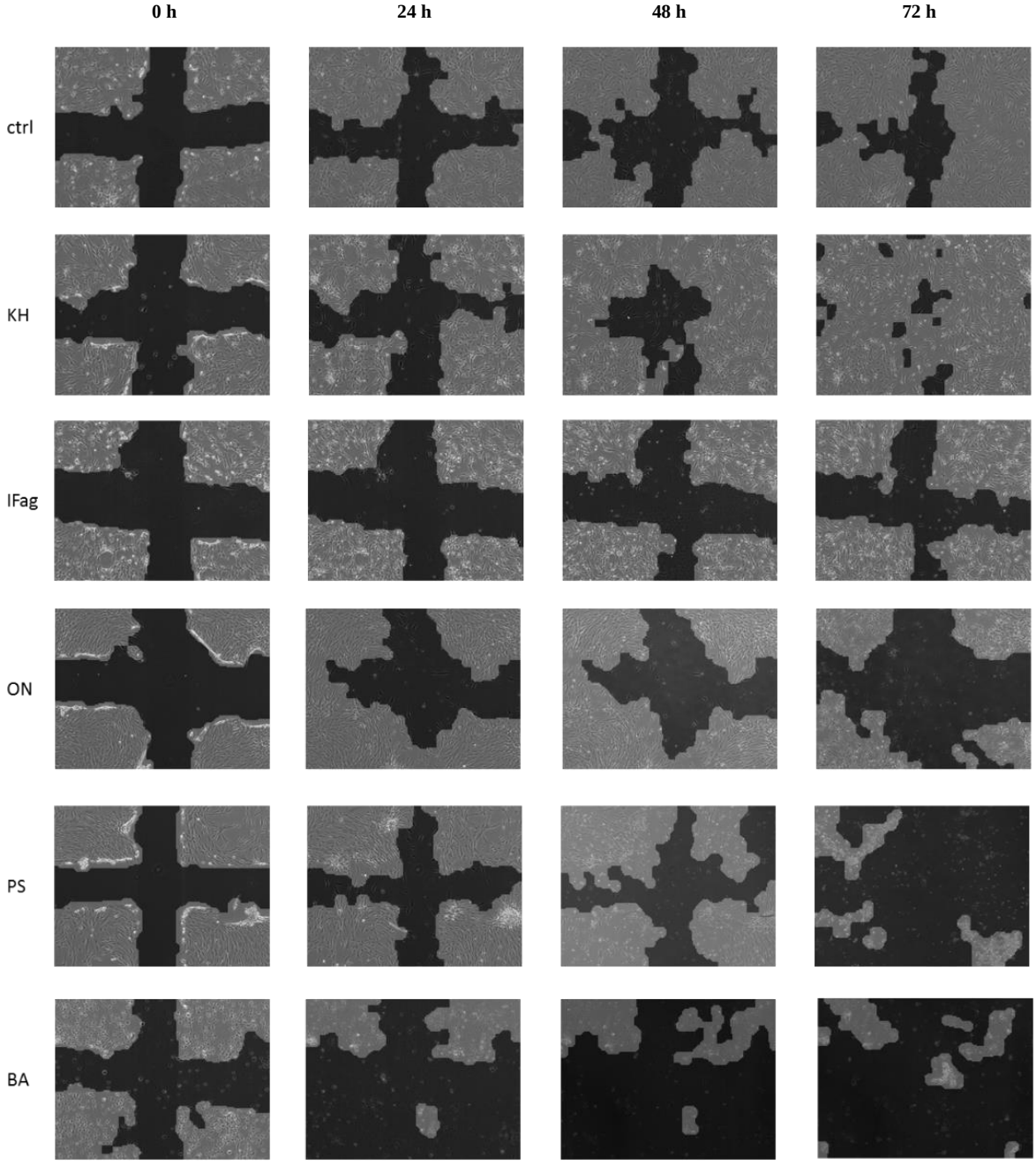
Scratched monolayers of NHDF in serum free medium were affected by 16 extracts BA, CD, CM, IFag, IT, KH, MM, ON, PG, PI, PP, PS, PhS, PrS, SG, SS at concentration 32 μg/ml and photographed by microscopy camera in time points 0, 24, 48, 72 h. Open area of the pictures was evaluated by TScratch software. Percentage of the open area was assessed and compared to the time 0 h. The reduction of open area indicated migration of cells. The scratch wound assay results of all extracts are referred in Graph 3. The examples of open area assesment by TScratch software are shown in Table 4.



Graph 3. ***The role of extracts in NHDF migration.*** NHDF were treated with 16 extracts (BA, CD, CM, IFag, IT, KH, MM, ON, PG, PI, PP, PS, PhS, PrS, SG, SS) at concentration 32 µg/ml for 72 h. Every 24 h cell migration was evaluated. Time 0 h served as a default value of the cell migration. Data are expressed as a mean ± SEM from at least 3 independent experiments.

The evaluated results showed cytotoxic effect (increase of open area more than 75%) particularly after 72 h of incubation in the case of 4 extracts (BA, PG, CM, CD), 6 extracts (PS, MM, PP, PI, SS, SG) demonstrated open area between 50-75% and 3 extracts (IFag, IT, ON) had no significant effect on cell migration even in the time point 72 h. On the contrary, 3 extracts (PrS, KH, PhS) intesively stimulated NHDF migration (values of open area less than 25%) particularly after 48 and 72 h of treatment. There was a time correlation in NHDF migration process (decrease of open area) in these samples.

Table 4. ***Illustrative examples of TScratch (CSELab, ETH Zurich) evaluation.*** The software detect an area with cells (grey) and define an open area with no cells (black). Percentage of the open area was assessed immediately after the treatment and every 24 h for 3 days. Reduction of the open area indicates migration of cells.



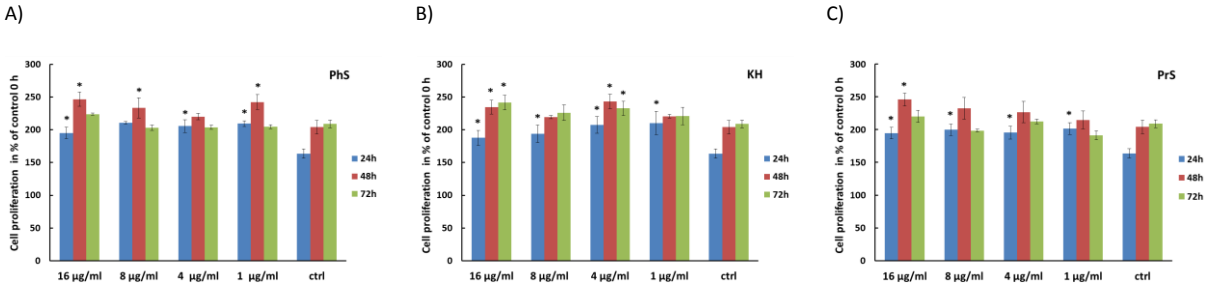
6.2 Selected extracts

Based on the screening data (see chapter 6.1), 3 extracts (PhS, KH, PrS) with the supporting effect on NHDF proliferation and migration have been chosen for further examination. The selected extracts were tested at lower concentrations 16, 8, 4 and 1 µg/ml, and statistically evaluated. Cell proliferation measurement and scratch wound assay were analysed by the same methods as in the screening part of this work.

6.2.1 NHDF proliferation analysis

6.2.1.1 Medium with FBS

NHDF were cultured in medium supplemented with 10% FBS and treated with selected extracts PhS, KH, PrS at concentrations 16, 8, 4 and 1 µg/ml for 3 days. The effect of extracts on NHDF proliferation was measured in time points 24, 48, 72 h and statistically analysed. The results are demonstrated in Graph 4.



Graph 4. ***Proliferation of NHDF treated with selected extracts PhS, KH, PrS in medium with 10% FBS.*** NHDF were affected by extracts A) PhS, B) KH, C) PrS at concentrations 16 µg/ml, 8 µg/ml, 4 µg/ml and 1 µg/ml. Cell proliferation was measured by DNA quantification in time points 24, 48, 72 h and was expressed as percentage of the untreated control in time 0 h. Data are presented as a mean ± SEM from 6 independent experiments. (*) indicates statistically significant differences of treated samples in comparison to the untreated control in each time point (Wilcoxon match paired test, $p \leq 0.05$).

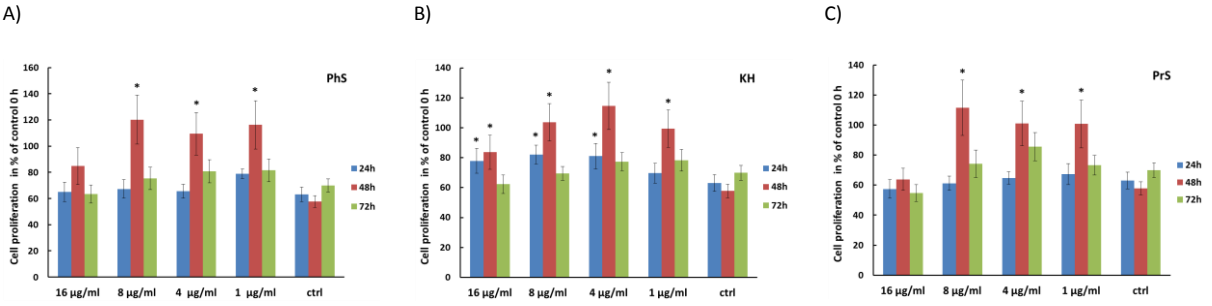
NHDF treated with the extracts PhS, KH, PrS showed statistically significant increase of cell proliferation particularly after 24 h of treatment. In the time point 24 h all selected extracts at all evaluated concentrations (except PhS 8 µg/ml) were statistically significant compared to the untreated control 24 h. After 48 h of incubation significantly stimulated

NHDF proliferation particularly extracts PhS (at concentrations 16, 8 and 1 µg/ml) and KH (at concentrations 16, 4 µg/ml), PrS increased cell proliferation only at concetration 16 µg/ml. On the other hand, in the time point 72 h significantly supported NHDF proliferation only extract KH at the concentration 16 and 4 µg/ml.

In summary, data analysis showed significant differences in the effect of extracts PhS, KH, PrS on NHDF proliferation compared to untreated controls 24, 48 and 72 h. The most significant values were demonstrated in time point 24 h and at concentration 16 µg/ml. On the contrary, after 72 h of treatment and at lower concentrations this effect was not observed any more. Generally, the best results provided extract KH, that stimulated NHDF proliferation in all the time points in particular at the concentration 16 µg/ml.

6.2.1.2 Serum free medium

NHDF were cultured in serum free medium and treated with selected extracts PhS, KH, PrS at concentrations 16, 8, 4 and 1 µg/ml for 72 hours. The effect of extracts on NHDF proliferation was measured and statistically analysed as in case of medium with 10% FBS. The results are referred in Graph 5.



Graph 5. ***Proliferation of NHDF treated with selected extracts PhS, KH, PrS in serum free medium.*** NHDF were affected by extracts A) PhS, B) KH, C) PrS at concentrations 16 µg/ml, 8 µg/ml, 4 µg/ml and 1 µg/ml. Cell proliferation was measured by DNA quantification in time points 24, 48, 72 h and was expressed as percentage of the untreated control in time 0 h. Data are presented as a mean ± SEM from 6 independent experiments. (*) indicates statistically significant differences of treated samples in comparison to the untreated control in each time point (Wilcoxon match paired test, $p \leq 0.05$).

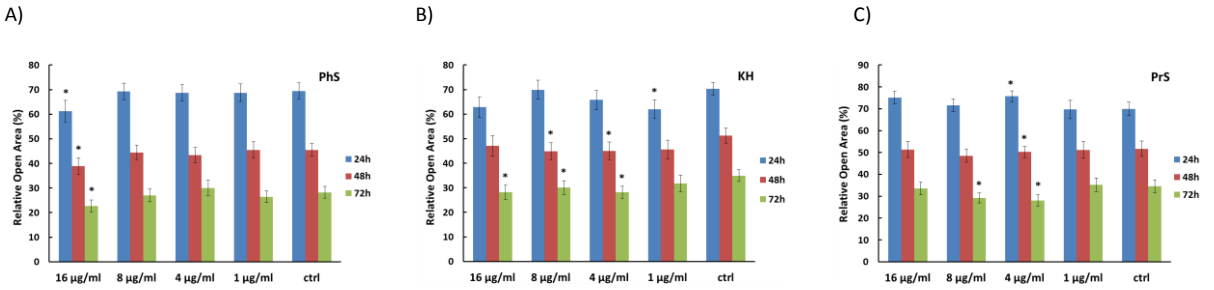
Analysis of NHDF treated with the extracts PhS, KH, PrS in serum free medium significantly induced cell proliferation particularly after 48 h of incubation. In the time point

48 h all extracts significantly stimulated NHDF proliferation at all evaluated concentrations (except PhS 16 μg/ml). On the other hand, after 24 h of treatment provided significant increase only extract KH (at concentrations 16, 8 and 4 μg/ml), and there was no significant difference in cell proliferation in the time point 72 h.

Comparing the results of NHDF proliferation in medium supplemented with 10% FBS to serum free medium, medium with FBS showed the most significant differences after 24 h, while in case of serum free medium this effect was observed after 48 h of the treatment. Approvingly, the best results showed extract KH, stimulating NHDF proliferation especially after 24 and 48 h of the treatment. Extract PhS provided also significant increase of cell proliferation in the time points 24 h (in medium with FBS), and 48 h (in medium with and without FBS). Extract PrS significantly stimulated NHDF proliferation after 24 h of incubation in medium with FBS, and after 48 h in serum free medium, respectively. On the contrary, after 72 h of treatment the cell proliferation has been reduced compared to 48 h values in the majority of cases. Statistically significant results after 72 h of treatment referred only extract KH in medium supplemented with 10% FBS.

6.2.2 NHDF migration analysis

Scratch wound assay was performed on extracts PhS, KH, PrS at concentrations 16, 8, 4 and 1 μg/ml. NHDF migration was monitored for 3 days and assessed every 24 h. Because of the significant effect of FBS on cell proliferation, scratch wound assay with serum free medium was applied. Scratched monolayers of NHDF treated with the extracts were evaluated by TScratch software and expressed as an open area in the time points 0, 24, 48 and 72 h. The reduction of open area indicated cell migration. Relative open area (a ratio of open area/open area in time 0 h) for statistical analysis was used. Results of NHDF migration treated with selected extracts are represented in Graph 6.



Graph 6. ***Migration analysis of NHDF treated with selected extracts.*** NHDF were affected by extracts A) PhS, B) KH, C) PrS at concentrations 16 µg/ml, 8 µg/ml, 4 µg/ml and 1 µg/ml for 72 h. Cell migration was evaluated by scratch wound assay and expressed as an open area. Relative open area was calculated as a ratio of open area in time 24-72 h to open area in time 0 h. Data are presented as a mean ± SEM from at least 6 independent experiments. (*) indicates statistically significant differences of treated samples in comparison to the untreated control in each time point (Wilcoxon match paired test, $p \leq 0.05$).

The evaluated data showed statistically significant effect of selected extracts on NHDF migration. The best results provided extract KH, significantly stimulating NHDF migration particularly after 72 h of treatment (at concentrations 16, 8, 4 µg/ml) and in the time point 48 h (at concentrations 8, 4 µg/ml). On the contrary, after 24 h of incubation reported significant difference only KH 1 µg/ml. Extract PhS significantly supported NHDF migration in all the time points at concentration 16 µg/ml, however this effect was not significant at lower concentrations. Extract PrS stimulated cell migration at concentration 4 and 8 µg/ml at all the time points, or at least after 72 h of treatment, respectively. Generally, during the time (0-72h) stimulation of NHDF migration (decrease of open area) by the all selected extracts was observed.

7 Discussion

Disrupted skin barrier is at high risk of infection and an access of other external noxae, leading to delayed and impaired wound healing, therefore it is nesessary to restore tissue integrity in prompt time (Shaw and Martin 2009; Delavary, van der Veer et al. 2011; Greaves, Ashcroft et al. 2013). For that reason various evidence-based active compounds even traditionally claimed medicines are used to shorten the time required for wound healing and minimize the undesired consequences (Shah and Amini-Nik 2017).

The current study was performed to find out wound healing properties of traditionally used plant species *Barringtonia asiatica, Cerbera manghas, Commelina diffusa, Inocarpus fagifer, Kleinhovia hospita, Mikania micrantha, Omalanthus nutans, Peperomia pellucida, Phymatosorus scolopendria, Piper graeffei, Premna serratifolia, Psychotria insularum, Schizostachyum glaucifolium* and *Solenostemon scutellarioides* collected on Samoa Islands for a purpose of wound healing, and scientifically analysed them. 16 plant extracts (BA, CD, CM, IFag, IT, KH, MM, ON, PG, PI, PP, PS, PhS, PrS, SG, SS) at various concentrations were tested on NHDF proliferation and migration *in vitro.*

Screening of all extracts showed diverse effects of the extracts on cell proliferation and migration. The best results of NHDF proliferation provided extracts at concentration 32 μg/ml. However, the higher conctrations had no significant effect or decreased cell proliferation with a concentration dependence. Meanwhile, at the concetration 32 μg/ml expressively reduced NHDF proliferation 3 extracts, at the highest concentration 512 μg/ml were cytotoxic 8 extracts or more. Cell proliferation assay was done on NHDF cultured in medium supplemented with 10% FBS and serum free medium. Comparing the results, NHDF cultured in medium with FBS referred distictively higher proliferation levels, that indicates a major influence of FBS on NHDF proliferation. Nevertheless, the principle of positive or negative effect of the extracts was preserved. Generally, the most significant increase of cell proliferation presented extracts PhS, KH, PrS, IFag, MM, IT and SG (medium with and without FBS) at concentration 32 μg/ml after 72 h of treatment.

Based on the proliferation results, screening of all extracts on NHDF migration was evaluated at the concentration 32 μg/ml. Because of the great impact of FBS, serum free medium in scratch wound assay was used to avoid excessive cell proliferation. The evaluated

data showed cytotoxic or nonsignificant effect on NHDF migration in majority of samples. On the other hand, extracts PhS, KH, PrS notably stimulated cell migration particularly after 72 h of incubation.

According to the screening data, 3 extracts with the most promising results of *in vitro* studies on NHDF migration and proliferation have been chosen for further examination. The best values demonstrated extracts PhS, KH and PrS, the other extracts have been from following studies exluded. Selected extracts were tested at lower concentrations 16, 8, 4 and 1 μg/ml and statistically analysed.

Data analysis showed a significance of selected extracts on NHDF proliferation. NHDF cultured in medium with 10% FBS demonstrated the most significant differences after 24 h, whereas in case of serum free medium this effect was observed after 48 h of treatment. As in the screening of all extracts, number of cells increased considerably slower in serum free medium. Approvingly in both medium, the best results showed extract KH, significantly stimulating NHDF proliferation particularly after 24 and 48 h of treatment in the majority of cases. Extracts PhS and PrS also provided increase of cell proliferation, however, the effect was not significant so often. On the contrary, after 72 h of treatment the cell proliferation have been reduced. Statistically significant results referred only extract KH in medium supplemented with 10% FBS.

The evaluated data showed statistically significant effect of selected extracts also on NHDF migration. As in the proliferation assay, the best results provided extract KH, significantly stimulating NHDF migration particularly after 48 h and 72 h of treatment. On the contrary, extract PhS and PrS significantly supported NHDF migration in all the time points at concentration 16 μg/ml and 4 μg/ml, respectively. However, this effect was not significant at other concentrations. The stimulatory effect on cell migration was time dependent with the maximum after 3 days of the treatment.

Scratch wound assay analysis confirmed the proliferation results, the selected extracts have a significant effect on wound healing properties. Cell proliferation and migration are two essential events necessary for wound healing, besides others during reepithelialization, so that proliferating fibroblasts at the wound site ensure an adequate number of cells to migrate and cover the wound surface (Delavary, van der Veer et al. 2011; Greaves, Ashcroft et al. 2013). Both aspects were proved in *in vitro* studies of cell proliferation and scratch wound assay at selected extracts. However, screening of all extracts provided satisfying results just in case of

32 μg/ml, higher concentrations 128 – 512 μg/ml were cytotoxic or decreased cell proliferation significantly in majority of samples.

The results discussed above established the scientific basis for traditionally used plants as wound healing agents. In the literature, it is possible to find some articles dealing with the tested plant species, their effects and chemical constituents. However, focus on the research of these Samoan plants greatly differs. Some of them are fairly enough explored, while the others are almost completely unknown. Our data confirmed certain results of other authors, e.g. cytotoxic effect of *Barringtonia asiatica*, that seeds are used as a fish poison or a vermifuge, and *Cerbera manghas* used in past as fatal poisonings (Ragasa, Espineli et al. ; Carlier, Guitton et al. 2014). Several plants such as *Kleinhovia hospita*, *Mikania micrantha*, *Peperomia pellucida*, *Premna serratifolia*, *Solenostemon scutellarioides* are relatively well described, regarding chemical compounds and biological uses. However, these plants are usually examined for their antioxidant, antimicrobial, antiinflammatory, hepatoprotective, or antitumor pharmacological activities (Selvam, Venkatakrishnan et al. 2012; Zhou, Zou et al. 2013; Dewanjee, Gangopadhyay et al. 2014; Biradi and Hullatti 2017; Djabir, Arsyad et al. 2017; Florence, Huguette et al. 2017; Li-Mei, Xu-Chao et al. 2017; Okoh, Iweriebor et al. 2017). Other plants such as *Inocarpus fagifer* is rarther known is an eatable legume, or *Schizostachyum glaucifolium* as a Polynesian bamboo (Larrue, Meyer et al. 2010; Huml, Mikšátková et al. 2016). Last but not least, plant species *Piper graeffei* is basically unknown for scientific world concerning the therapeutical use.

Generally, wound healing properties of the tested extracts have been not fully studied with a systematic manner. This study contributes to better understanding the wound healing effects of these extracts, regarding selected extracts confirmed significant stimulation of *in vitro* cell proliferation and migration. Further examination should be concerned on specific identification of the active compounds and deep investigation of biological effects, since major knowledge is always required for right understanding and progress in searching for novel active principles, that leads in potential drug development.

8 Conclusion

This study demostrated various effect of tested extracts on *in vitro* NHDF proliferation and migration. Screening of all extracts showed diverse outcomes with an apparent concentration dependence. The best results of screening data provided extracts PhS, KH and PrS at concetration 32 µg/ml. These extracts were subjected to further examination at lower concentrations and statistically analysed. The selected extracts significantly stimulated NHDF proliferation and migration, while the most significant differences performed extract KH. Extracts PhS and PrS also induced cell proliferation and migration, however, the signicant effect was not observed so often.

Although a long time traditional use of idigenous cultures for wound healing and promising results of selected extracts in this study, there is a need of further research and precise characterization to guarantee the wound healing properties and safe use of these phytochemicals, leading to evidence-based therapeutical application as natural agents for treatment of wounds.

9 Abbreviations

αSMA	α-smooth muscle actin
ADP	Adenosine diphosphate
Ang-1	Angiopoietin 1
BA	*Barringtonia asiatica* extract
CD	*Commelina diffusa* extract
CDK	Cyclin-dependent kinase
CM	*Cerbera manghas* extract
CREB	cAMP response element-binding protein
CTGF	Connective tissue growth factor
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GTP	Guanosine triphosphate
IGF-1	Insuline-like growth factor 1
IFag	*Inocarpus fagifer (bark)* extract
IFN-γ	Interferon γ

IL	Interleukin
IT	*Inocarpus fagifer (leaves)* extract
JNK	Jun kinase
KGF	Keratinocyte growth factor
KH	*Kleinhovia hospita* extract
MAPK	Mitogen-activated protein kinase
MM	*Mikania micrantha* extract
mTOR	Mammalian target of rapamycin
NHDF	Normal human dermal fibroblast
NSAIDs	Nonsteroidal anti-inflammatory drugs
ON	*Omalanthus nutans* extract
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PG	*Piper graeffei* extract
PhS	*Phymatosorus scolopendria* extract
PI	*Psychotria insularum* extract
PI3K/AKT	Phosphoinositide 3-kinase/ Protein kinase B
PMN	Polymorphonuclear leukocytes
PP	*Peperomia pellucida* extract
PrS	*Premna serratifolia (bark)* extract
PS	*Premna serratifolia (leaves)* extract
SEM	Standard error of mean
SG	*Schizostachyum glaucifolium* extract
SS	*Solenostemon scutellarioides* extract

ROCK	Rho-associated protein kinase
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
t-PA	Tissue plasminogen activator
u-PA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

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13 References

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